UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

METABOLISMO DE LOS FENILPROPANOIDES Y PROTEÍNAS RELACIONADAS CON LA PATOGÉNESIS EN EL MECANISMO DE RESPUESTA DE UVA DE MESA ("VITIS VINIFERA" L. CV. CARDINAL) A ELEVADAS CONCENTRACIONES DE CO2 Y BAJAS TEMPERATURAS

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Irene Romero de la Fuente

Bajo la dirección de las doctoras
Mª Teresa Sánchez-Ballesta y Carmen Merodio Moreno

Madrid, 2009

- **ISBN: 978-84-692-1103-8**

UNIVERSIDAD COMPLUTENSE

FACULTAD DE CIENCIAS BIOLOGICAS

Metabolismo de los fenilpropanoides y proteínas relacionadas con la patogénesis en el mecanismo de respuesta de uva de mesa (*Vitis vinifera* L. cv. Cardinal) a elevadas concentraciones de CO_2 y bajas temperaturas

TESIS DOCTORAL

IRENE ROMERO DE LA FUENTE

MADRID, 2008

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Metabolismo de los fenilpropanoides y proteínas relacionadas con la patogénesis en el mecanismo de respuesta de uva de mesa (*Vitis vinifera* L. cv. Cardinal) a elevadas concentraciones de CO_2 y bajas temperaturas

Memoria presentada por
IRENE ROMERO DE LA FUENTE
para optar al grado de
DOCTORA en CIENCIAS BIOLÓGICAS

Directoras
DRA. Mª TERESA SANCHEZ-BALLESTA
DRA. CARMEN MERODIO MORENO
Científicos Titulares del CSIC

Madrid, 2008

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

INSTITUTO DEL FRÍO
C/ José Antonio Novais, 10
28040, Madrid
Tlf: 91-5492300

Mª Teresa Sánchez Ballesta, Doctora en Farmacia, contratada del Consejo Superior de Investigaciones Científicas adscrito al Instituto del Frío de Madrid

Certifica: que Dª Irene Romero de la Fuente ha realizado bajo su codirección el trabajo con el título: **"Metabolismo de los fenilpropanoides y proteínas relacionadas con la patogénesis en el mecanismo de respuesta de uva de mesa (*Vitis vinifera* L. cv. Cardinal) a elevadas concentraciones de CO_2 y bajas temperaturas"**, que presenta para optar al grado de Doctor en Biología

Para que conste a los efectos oportunos, firma el presente certificado en Madrid a 14 de diciembre de 2007.

Mª Teresa Sánchez Ballesta

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

INSTITUTO DEL FRÍO
C/ José Antonio Novais, 10
28040, Madrid
Tlf: 91-5492300

Carmen Merodio Moreno, Doctora en Biología, científico titular del Consejo Superior de Investigaciones Científicas adscrito al Instituto del Frío de Madrid

Certifica: que Dª Irene Romero de la Fuente ha realizado bajo su codirección el trabajo con el título: **"Metabolismo de los fenilpropanoides y proteínas relacionadas con la patogénesis en el mecanismo de respuesta de uva de mesa (*Vitis vinifera* L. cv. Cardinal) a elevadas concentraciones de CO_2 y bajas temperaturas"**, que presenta para optar al grado de Doctor en Biología

Para que conste a los efectos oportunos, firma el presente certificado en Madrid a 14 de diciembre de 2007.

Carmen Merodio Moreno

Quiero mostrar mi más sincero agradecimiento a todas las personas que han contribuido directa o indirectamente a la realización de esta tesis. Habéis sido muchos, así que espero no dejarme a nadie en el tintero….

A mis directoras de tesis, Maite y Carmen, por la oportunidad que me habéis brindado al confiar en mí para que llevara a cabo este trabajo.

Maite, muchas gracias por haberme enseñado tanto en este "corto" periodo de tiempo; por tu entrega, paciencia y dedicación. Ha sido un placer poder compartir contigo tan buenos momentos a lo largo de estos cuatro años: en el laboratorio, en los cafés, en quedadas varias, Congresos…. y estoy segura de que serán muchos más! Gracias porque además de ser un ejemplo a seguir a nivel profesional, puedo decir que eres mi amiga.

Carmen, muchas gracias por tu apoyo y ayuda en la elaboración de esta tesis. Me he sentido muy acogida y arropada en el grupo desde el primer día que llegué. Gracias por tus consejos, conocimientos y por los buenos momentos que hemos compartido.

También quiero dar las gracias de manera especial a María I. Escribano; sin tu ayuda esta tesis habría costado bastante más. Gracias por los consejos, conversaciones y momentos agradables de estos años.

Además, a todos los que habéis trabajado en el grupo coincidiendo con mi estancia:

A "Oskir", por tu ayuda en el laboratorio, por tu compañía durante este caminar, por los ratos divertidos que hemos compartido, risas, bromas, cenas y bailes asincrónicos…y, principalmente, por ser mi amigo. Hemos pasado muchos momentos juntos y has estado en los buenos (la mayoría), y en los no tan buenos…Gracias!

A Carlos ("Charly"), porque aunque has llegado el último año, hemos trabajado codo a codo muchos días y ha sido un tiempo vivido muy intensamente. Muchas gracias por tu ayuda en el laboratorio y por las anécdotas y risas que hemos compartido; aunque este año los resultados se hicieron esperar…al final salieron!!! (Barabarabarabá!).

A todos los que han pasado por este laboratorio: Roberto, por tu ayuda y apoyo los primeros años cuando aparecí por el laboratorio; Helena, Raquel, Patricia y Paloma. Y a los técnicos: Delfina, Oscar B., Olga, Miguel A. y Fernando.

Gracias, porque la buena compañía es un factor muy importante para que las cosas salgan adelante.

A la maja remaja (Sonia), por amenizar los cafés matutinos y demás fiestas de guardar. Gracias por la oportunidad que me diste de asistir al curso en Kiel (fue toda una experiencia!); por ser tan auténtica y por tus cuidados: ¡Viva el hornazo! (Aunque a veces vayas de dura…te tengo "calá").

A María Hidalgo, gracias por tu apoyo. Aunque has venido hace poco ha dado tiempo de sobra para pasar buenos ratos y compartir preocupaciones y alegrías. Y espero que siga siendo así….

A los "compis" de la sala de becarios; sois muchos los que habéis pasado por la salita, pero especialmente os agradezco a los actuales la paciencia y el apoyo que me habéis dado en estos meses de "enclaustramiento". A Sara, por tu alegría, tus ánimos y tu apoyo. Me alegro que nos volviésemos a encontrar aquí después de los años de facultad (¿Quién nos lo iba a decir, eh?). A las vecinas de enfrente: Tati, Tati, Tati (con musiquita, claro) y Ailén

(por surtirme de chicles), porque me habéis hecho pasar muy buenos momentos. A Mariví, Pilar y Pedro, por vuestros buenos consejos y ánimos.

A todos los investigadores y compañeros del Departamento de Ciencia y Tecnología de Productos Vegetales, a los que estuvieron y a los que actualmente están; especialmente a Cris, Patri, Eva, Inés, Clara y "Fer" con los que he compartido buenos momentos. Y al Departamento de Ciencia y Tecnología de Productos Lácteos; por vuestra amabilidad, y generosidad al compartir equipos y material que han facilitado la realización de esta tesis. A Teresa Requena, Marta, Raquel, Tomás y Cris.

A los actuales "compis" de sala de Maite y Sonia (extensión 284), a la que tanto he llamado y visitado: Luis, Nacho, Sonia, Carmen. Gracias por vuestra paciencia.

Al Instituto del Frío donde he podido llevar a cabo este trabajo; al CSIC por el soporte económico; y al Departamento de Fisiología Vegetal de la Facultad de Biología (UCM).

A todos mis amigos, por animarme y estar preocupados por mi incierto porvenir desde que me lancé de cabeza en este difícil, y a la vez gratificante, mundo de la investigación.

A mis amigas del cole: Isa, Miry (por extensión a Alfon) y Ro, a las que conozco "de toda la vida", como se suele decir, y con las que he viajado y disfrutado tanto estos años: de marcha, en conciertos, comiendo (cómo nos gusta, eh?)… A Natalia y Melania, por los cafés y buenos momentos de charla que hemos tenido. A Sandra (y familia), porque sé que estáis más cerca que la distancia física que nos separa.

A mis compañeras de la Facultad. Especialmente a "Esthercita", por tu amistad. ¡Anda que no hemos pasado horas en este Campus! Gracias por los buenos momentos que hemos compartido, y también por estar en los momentos menos buenos, que de todo hay en la vida. Sé que aunque haya etapas que nos veamos menos podremos contar siempre la una con la otra. Gracias por tu apoyo y ojalá podamos celebrar muchos éxitos en breve: ¡Ánimo con las Oposiciones!

A mis amigos de la parroquia, a mis "chavales" del grupo y especialmente a Giuseppe y Mane. Gracias por vuestro cariño y por apoyarme siempre.

A Ani, por tu alegría, por tu ayuda con el inglés y por tus sabios consejos.

Y casi finalizando, pero sin restar importancia ni emoción, a mis amigos del pueblo, a Alberto, y a mi peña de Montalbanejo. Gracias por vuestros ánimos. Con especial dedicación a Lore (gracias por tu amistad incondicional en todos estos años), Javi (Farru), Ana, Raquel, Amparo, David (Pati), Alicia, Alex (Conejo), David (Liebre) y demás…. Que sois muchos, "copón"!!! A todos.

Ahora sí, para terminar, quiero agradecer A TODA MI FAMILIA el cariño y apoyo que me ha dado siempre; y, sobre todo, durante el periodo de la tesis. Especialmente a los que ya no nos acompañan físicamente, pero que siento muy cerca, a mi padre y a mi abuelo Abel. Y a las personas con las que comparto el día a día: a mi madre, por mimarme tanto, por confiar en mis proyectos y porque sé que puedo contar contigo y será así siempre. A Fede, por tu apoyo incondicional y generoso; por hacerme reír en los momentos "regulines" y por los muchos y buenos momentos que hemos compartido (¡y los que nos quedan por vivir!). Y a Alex, mi hermanito querido (¡"Ratón"!), por aguantar "mis cosillas", que no es poco! GRACIAS.

A mi madre y a mi hermano

A Fede

“El futuro pertenece a los que creen
en la belleza de sus sueños”

(Eleanor Roosevelt)

ABREVIATURAS

aa	Aminoácidos
ABA	Ácido abscísico
ACC	1-aminociclopropano-1-carboxilato
AFP	Antifreeze proteins
AOX	Alternativa Oxidasa
APX	Ascorbato peroxidasa
CA	Atmósfera controlada
CAT	Catalasa
CBD	Cysteine-rich chitin-Binding Domain
CBF	Cysteine Binding Factor
CHS	Chalcona sintasa
CoA	Coenzima A
COR	Cold –Regulated
HR	Humedad relativa
HSP	Heat Shock Proteins
HPLC-DAD-MS	Cromatografía de líquidos de alta eficacia-Detección Diodo Array-Espectrometría de Masas
JA	Ácido jasmónico
LEA	Late Embriogenesis Abundant
LOX	Lipooxigenasa
nsLTP	Proteínas transportadoras de lípidos no específicas
MAP	Empaquetamiento en atmósferas modificadas
MAPK	Mitogen-Activated Protein Kinase
MeJA	Metil Jasmonato
MeSA	Metil Salicilato
mRNA	RNA mensajero
PAL	L-fenilalanina amonio-liasa
Pb	Pares de bases
PEP	Fosfoenol piruvato
POD	Peroxidasa
PPO	Polifenol oxidasa
PR	Pathogenesis related
Put	Putrescina
ROS	Reactive oxygen species
SA	Ácido salicílico
SAR	Sistema de resistencia adquirida
SOD	Superóxido dismutasa
Spd	Espermidina
Spm	Espermina
STS	Estilbeno sintasa
UV	Ultravioleta

INTRODUCCIÓN

Introducción

El empleo de tecnologías no contaminantes es necesario para dinamizar la oferta de productos hortofrutícolas frescos en las diferentes fases de producción, cosecha y postcosecha. En este sentido, es necesaria su incorporación en la fase de producción permitiendo una mayor biodiversidad, al mismo tiempo que se mantienen los rendimientos y se mejora el control fitosanitario reduciendo el uso de productos químicos. Una vez obtenidos productos frescos de calidad, los procesos de manejo postcosecha y la logística de la comercialización para mantener la calidad, garantizar la seguridad y reducir las pérdidas de producto, constituyen objetivos fundamentales en las actuales investigaciones en fisiología y tecnología postcosecha.

En el caso concreto de la uva de mesa, muy susceptible a las pérdidas de agua y a la infección fúngica causada principalmente por *Botrytis cinerea* Pers. durante su conservación a bajas temperaturas, las tecnologías postcosecha se centran en evitar la proliferación del hongo y en mantener los parámetros de calidad de la uva durante su periodo de vida útil.

1. CARACTERÍSTICAS Y PARÁMETROS DE CALIDAD DE UVA DE MESA

La uva o grano de uva es el nombre que recibe el fruto que crece formando racimos de la vid común. Pertenece al género *Vitis* de la familia Vitáceas, que incluye unas 600 especies de arbustos, por lo general trepadores y que producen frutos en baya. Dentro del género *Vitis* se incluyen unas 20 especies cultivadas por sus frutos y algunas por sus hojas que se consumen como verdura. Más del 95% de la producción total de uva proviene de la vid europea que pertenece a la especie *Vitis vinifera* L. y cuyas variedades se destinan principalmente a vinificación.

La composición de la uva varía según se trate de uvas blancas o tintas. En ambas destacan dos tipos de nutrientes: los azúcares (siendo la glucosa y la fructosa más del 99%

de los hidratos de carbono en el zumo de uva y constituyendo del 12 al 27% del peso fresco de la baya madura) (Hofacher et al., 1976), y las vitaminas (principalmente ácido fólico y vitamina B6). La fracción ácida de las uvas está formada principalmente por los ácidos tartárico y málico, constituyendo alrededor del 90% de la acidez total (Winkler et al., 1974). Otros ácidos orgánicos encontrados en proporciones variables pero siempre a bajas concentraciones son los ácidos cítrico (5-10% de la acidez total), succínico, fumárico, acético, glicólico, láctico, aconítico, quínico, siquímico y mandélico (Ruffner, 1982). Entre los minerales, el potasio es el más abundante y se encuentra en mayor cantidad en uva tinta; mientras que el magnesio y el calcio están en cantidades moderadas y son más abundantes en uva blanca. También abundan compuestos con reconocidas propiedades beneficiosas para la salud, tales como flavonoides, antocianos, y taninos, responsables al mismo tiempo del aroma, color, y textura característicos de estos frutos.

La uva de mesa es un fruto importante en la dieta mediterránea que se consume casi todo el año. El cultivo de uva de mesa en España supone un 2,6 % del total de uva cultivada. Según el Instituto Nacional de Estadística, la superficie agrícola destinada a la producción de uva de mesa en España en el año 2005 fue de 26445 Ha.

Las uvas, como otros frutos no climatéricos, no maduran después de cosechadas; alcanzando el óptimo de aceptabilidad en apariencia, sabor y textura mientras están en la vid. Su índice de madurez se ha basado, tradicionalmente, en el contenido de sólidos solubles (ºBrix) y también en el valor de acidez titulable, así como en la relación azúcares/ácidos de las bayas (Guelfat-Reich y Safran, 1971). La apariencia es determinada principalmente por el color, especialmente en variedades coloreadas, en las que existen requerimientos mínimos que varían con la variedad y el grado estándar. El grado estándar designa el porcentaje de bayas por racimo, el cual debe mostrar una intensidad mínima de color y cobertura (Nelson, 1979). La cera de la superficie de las bayas es un factor de

calidad muy importante en la uva de mesa; la manipulación postcosecha en ocasiones destruye esta cera, haciendo que la piel esté más brillante que el efecto deseable que aporta la cera. El aroma es un carácter importante y complejo de la calidad de uva ya que es una mezcla de cientos de compuestos volátiles diferentes sintetizados durante la maduración (Gunata et al., 1985a,b; Strauss et al., 1986). Aunque los compuestos precursores de estas sustancias se encuentran en las hojas (Gunata et al., 1986), la síntesis y evolución del aroma tiene lugar en las bayas; y de hecho, las sustancias aromáticas están localizadas principalmente en la piel de las mismas (Winkler et al., 1974).

Las uvas están sujetas a serias pérdidas de vapor de agua, que causan desecamiento y oscurecimiento del raquis, roturas e incluso marchitamiento de las bayas y pérdida de peso del racimo (Nelson, 1979). Por esto, los racimos deben ser enfriados lo antes posible después de la cosecha. En este contexto, el "sudado" de la uva, como resultado de cambios drásticos y rápidos en la temperatura durante el almacenamiento, causa serios problemas asociados a la podredumbre por *Botrytis cinerea,* que es junto a las pérdidas de agua, uno de los principales responsables de las pérdidas postcosecha que determinan la vida útil de la uva de mesa (Lavee y Nir, 1986; Crisosto et al., 2002).

La uva de mesa cv. Cardinal se caracteriza por su color rojo violeta, presenta una pulpa jugosa y aromática y piel de espesor medio. Su maduración es temprana, variando desde mediados de julio a finales de agosto, dependiendo de las zonas. Aunque su origen es californiano, es una variedad muy cultivada en todo el mundo. En España, se cultiva en formas libres mayoritariamente en las provincias de Valencia, Alicante y Castellón, donde se obtienen unas producciones de aproximadamente 6000-7000 kg/Ha.

2. MECANISMOS IMPLICADOS EN LA INFECCIÓN Y RESPUESTA DEL FRUTO AL ATAQUE FÚNGICO

2.1. Factores intrínsecos implicados en el ataque fúngico

El hongo *Botrytis cinerea* es un patógeno necrotrófico que coloniza tejidos senescentes o muertos, pero debido a que es capaz de infectar también a bajas temperaturas, puede producir importantes pérdidas económicas durante la conservación en frío (Mansfield y Hutson, 1980).

Al igual que otros patógenos, *B. cinerea* utiliza 3 vías principales para penetrar en el tejido del huésped: (i) a través de aberturas naturales tales como lenticelas, finales de tallos e interfases pedicelo-fruto; (ii) por grietas directas de la cutícula del huésped que pueden ocurrir en cualquier momento durante el periodo de crecimiento y desarrollo del fruto; (iii) y a través de heridas causadas por agentes bióticos y/o abióticos durante el almacenamiento (Prusky y Lichter, 2007).

Los procesos de penetración pueden pasar desapercibidos para el huésped, o pueden promover rápidas señales de defensa que resultan en la inducción de compuestos de defensa para limitar el desarrollo del hongo. El periodo desde la infección hasta la activación del desarrollo del hongo y expresión de los síntomas se designa etapa quiescente (Prusky, 1996). Esta etapa podría ser el resultado de una respuesta localizada del huésped que se asocia a menudo con un "estallido oxidativo", que da lugar a la producción de especies reactivas de oxígeno (ROS). La producción de ROS en las células ha sido también relacionada con los procesos de defensa de las plantas (Brisson et al., 1994). Se ha observado que tanto la inoculación del pericarpo de aguacate con *Colletotrichum gloeosporioides* como el tratamiento de cultivos celulares de aguacate con el elicitor de la pared celular de *C. gloeosporioides*, aumentaron la producción de ROS (Beno-Moualem y

Prusky, 2000). También, se han descrito cambios en el pH tisular de los frutos asociados a los procesos de maduración e infección por patógenos (Wang et al., 2004). Los hongos son capaces de alcalinizar el ambiente debido a la secreción activa de amonio liberado en las reacciones de proteolisis y desaminación de los aminoácidos (Eshel et al., 2002). En el caso de *B. cinerea* la acidificación de los tejidos del huésped es debido a la secreción de ácidos orgánicos, concretamente de ácido oxálico (Rollins y Dickman, 2001; Manteau et al., 2003).

La transición de la etapa biotrófica a la necrotrófica-saprofítica parece estar relacionada con factores que son modulados a nivel intracelular y que están afectados por los nutrientes y el pH del ambiente. Cada uno de los compuestos secretados (ácidos orgánicos o amonio) juegan un papel fisiológico crucial en la iniciación del desarrollo necrotrófico. Existen varias especulaciones acerca de las vías por las cuales la secreción de ácidos orgánicos aumenta los centros de virulencia. El oxalato podría debilitar al huésped, al ser tóxico para él, lo que facilitaría su invasión. Asimismo, se ha propuesto como hipótesis que la quelación de calcio de la pared celular por parte del oxálico y ácidos glucónicos debilita la pared celular del huésped y facilita su penetración (Hadas et al., 2007). Finalmente, la secreción de oxalato podría suprimir la generación de ROS y las respuestas de defensa asociadas, contribuyendo de este modo a la activación del modo de desarrollo necrotrófico (Cessna et al., 2000).

2.2. Factores ambientales o extrínsecos implicados en el ataque por *Botrytis*. Temperatura y humedad.

Estudios acerca de la viabilidad del hongo almacenado a bajas temperaturas demostraron que, mientras que la agresividad patogénica de los conidios de *B. cinerea* almacenados a -80ºC era casi igual a la de los conidios frescos, ésta decrecía según

aumentaba la temperatura de almacenamiento (Gindro y Pezet, 2001). Puesto que la actividad de estos microorganismos continúa a bajas temperaturas, los frutos son susceptibles al progreso de la infección durante su conservación frigorífica.

La humedad relativa (HR) también afecta al desarrollo de la infección, pero su efecto es dependiente de la temperatura. Estudios con flores Freesia infectadas por *B. cinerea* a distintas condiciones de temperatura y HR mostraron que la infección fue severa en las plantas almacenadas a 5, 12 y 20ºC a HR del 100%; mientras que no hubo lesiones cuando fueron almacenadas a HR de 80 a 90% a 5ºC ni a 20ºC (Darras et al., 2006). Por consiguiente, la temperatura de conservación y humedad relativa ambiental son parámetros esenciales a tener en cuenta a la hora de evitar la supervivencia y multiplicación de estos hongos.

2.3. Factores endógenos implicados en la respuesta a la infección. Respuestas fisiológicas, bioquímicas y moleculares inducidas en las plantas en respuesta al ataque fúngico.

Las plantas responden al ataque por patógenos mediante la inducción de diferentes mecanismos de defensa. Estos incluyen respuestas específicas que operan a través de la acción de moléculas señal tales como el ácido salicílico (SA), el ácido jasmónico (JA), o el etileno, y que generan la acumulación de proteínas relacionadas con la patogénesis (PRs, del inglés *Pathogenesis-Related proteins*), fitoalexinas, u otros compuestos fenólicos (Elad, 1997; Dong, 1998; Feys y Parker, 2000). Se ha observado un aumento en el contenido de la fitoalexina resveratrol en hojas y frutos infectados con el hongo (Jeandet et al., 1995). Asimismo, se ha observado la acumulación de barreras mecánicas como carbohidratos y glicoproteinas ricas en hidroxiprolina entre las paredes celulares para limitar la invasión de las hifas del hongo (Showalter et al., 1985).

El ácido abscísico (ABA) es una de las hormonas relacionadas con la interacción planta-patógeno. Se ha descrito que mutantes de tomate (*sitiens*) (*Lycopersicon esculentum* Mill. Cv Moneymarker) con niveles de ABA reducidos son mucho más resistentes al ataque por *B. cinerea* que las plantas de tipo salvaje (Audenaert et al., 2002). Por otro lado, estudios realizados en tomate con aplicaciones de ABA exógeno mostraron que aumentos en el contenido de ABA por encima del nivel basal no incrementaron la susceptibilidad de los frutos a *B. cinerea* (Achuo et al., 2006).

En *Arabidopsis*, se ha descrito que el pretratamiento con metil jasmonato (MeJA) causó una eficiente reducción de porcentaje de podredumbre causado por *Alternaria brassicicola*, *B. cinerea* o *Plectosphaerella cucumerina* (Thomma et al., 2000). Asimismo, mutantes de *Arabidopsis* (*npr1*), que presentaban alterada la resistencia frente a patógenos bacterianos y fúngicos, mostraron defectos en la acumulación, percepción o señal de transducción del SA. Los resultados mostraron que la resistencia local frente a *B. cinerea* requiere señales mediadas por etileno, jasmonato y SA (Ferrari et al., 2003).

A nivel molecular, se ha observado la acumulación de distintas proteínas PRs. Algunas de ellas son enzimas hidrolíticas capaces de degradar componentes de la pared celular de patógenos, como quitinasas o β-1,3-glucanasas. Los niveles de estas proteínas o su correspondientes actividades, incrementan en hojas de uva infectadas con *B. cinerea* (Derckel et al., 1999). También se han detectado incrementos en los niveles de distintos mRNAs que codifican PRs en hojas y/o granos como respuesta a los agentes causales de la podredumbre gris (Busam et al., 1997a) así como durante el proceso de desarrollo de la uva (Davies y Robinson, 2000). En fresa inoculada con *Colletotrichum*, se observó que la inducción de la expresión de genes de quitinasa de clase II estaba regulada diferencialmente en respuesta al patógeno (Anwar y Ding, 2004). La quitinasa CHV5, purificada de uva y que presenta gran homología con varias quitinasas de clase IV, parece

conferir una mayor protección frente a patógenos que la mayoría de las quitinasas estudiadas y fue capaz de inhibir hasta un 50% la germinación de los conidios de *B. cinerea* con una concentración de 7.5 μg mL^{-1} de proteína (Derckel et al., 1998). Por otro lado, en uvas Chardonnay infectadas con *Uncinula necator* se observó un aumento en los niveles de una proteína tipo taumatina (VvTL2) en comparación con las uvas no infectadas (Girbau et al., 2004).

Además de estas proteínas hidrolíticas, también se han observado aumentos en los niveles de expresión de los genes que codifican proteínas relacionadas con el metabolismo de los fenilpropanoides tales como la L-fenilalanina amonio-liasa (PAL) y la estilbeno sintasa (STS) (Sparvoli et al., 1994) como respuesta al ataque por patógenos en la vid. Bézier et al. (2002), además de analizar algunas PRs y la expresión de los genes que codifican las enzimas PAL y STS, clonaron y caracterizaron el gen que codifica la proteína inhibidora de la poligalacturonasa (PGIP), cuyos niveles de expresión se indujeron en respuesta a la infección por *B. cinerea*. PGIP inhibió la actividad de la poligalacturonasa secretada por los hongos fungitóxicos y aumentó la estabilidad de los oligalacturónidos activos así como el elicitor *in vitro* (Cervone et al., 1989).

Se ha investigado el papel de los oligogalacturónidos (OGA), compuestos liberados de las pectinas de la pared en las células heridas, para inducir las respuestas de defensa en vides y su protección frente a *B. cinerea*. Estudios cinéticos con células de *Vitis* mostraron que los OGA inducen una rápida y transitoria generación de peróxido de hidrógeno (H_2O_2), seguida de un aumento en la expresión de genes que codifican proteínas implicadas en la ruta de los fenilpropanoides, y estimulan las actividades de quitinasa y β-1,3-glucanasa, lo que podría estar relacionado con una mayor respuesta de defensa de la planta frente al hongo (Aziz et al., 2004).

También se ha estudiado el papel del calcio en el mantenimiento de la estabilidad e integridad de la pared celular así como de la membrana plasmática en la piel de uva por medio de la quelación de sustancias pécticas (Chardonnet et al., 1997). Además, se ha observado que la aplicación exógena de calcio incrementa su contenido en bayas y aumenta la resistencia a *Botrytis* en racimos conservados a bajas temperaturas (Miceli et al., 1999). La mayor resistencia a *B. cinerea* se atribuyó a la participación de los iones de calcio en la estabilización de la estructura de la pared celular en hojas de *Vitis* (Bowen et al., 1992).

3. CONSERVACIÓN DE FRUTOS Y HORTALIZAS A BAJAS TEMPERATURAS

La conservación a bajas temperaturas, por encima de las de congelación, es una de las técnicas más utilizadas para mantener la calidad y prolongar el periodo de vida útil de frutos y hortalizas: reduciendo la tasa de respiración y disminuyendo tanto la pérdida de agua por transpiración como la producción de etileno y el desarrollo de microorganismos. Sin embargo, debido a la alta susceptibilidad de determinados productos hortofrutícolas de origen tropical y subtropical al daño por frío, o bien a consecuencia de la aplicación de temperaturas inferiores a la óptima de conservación (subóptimas) en frutos tolerantes a las bajas temperaturas, se pueden provocar importantes pérdidas de calidad del producto durante su conservación (Morris 1982; Jackman et al., 1989). La naturaleza y severidad de los síntomas de daño por frío depende de la especie, variedad, tejido y estado de desarrollo o maduración, así como de la intensidad y duración de la exposición a las condiciones de estrés (Salveit y Morris, 1990). En uva de mesa, el empleo de temperaturas próximas a 0ºC, puede provocar en algunas variedades disfunciones fisiológicas que conducen a una pérdida de calidad. Uvas cv. Jingxiu almacenadas durante 3 días a -2ºC, presentaron daños en la ultraestructura de las células del pericarpo, desórdenes en la lamela estromal de los

cloroplastos o plastidios, pérdida de paredes celulares y alteraciones en la membrana nuclear (Zhang et al., 2005). Además, en estas condiciones, el periodo de conservación de la uva de mesa es limitado debido a su gran susceptibilidad al ataque fúngico.

3.1. Respuestas fisiológicas, bioquímicas y moleculares asociadas a la conservación a bajas temperaturas.

A pesar de que existen distintas teorías en la bibliografía, la mayoría de los autores están de acuerdo en que el proceso de daño por frío debería considerarse en dos etapas: una primera etapa en que el daño ocurriría de forma inmediata por la exposición a una temperatura crítica, y posteriormente una segunda en la que se originarían otros procesos secundarios dependientes del tiempo y de la temperatura de exposición que conducirían a los síntomas visibles de la lesión (Raison y Lyons, 1986). Las diferencias en lípidos y ácidos grasos entre las distintas plantas determinarían sus diferentes susceptibilidades, de forma que el cambio de fase de un estado líquido-cristalino a un estado sólido-gel en los lípidos de membrana alteraría tanto la permeabilidad como la energía de activación de las enzimas unidas a membrana, explicando así las alteraciones metabólicas de estas células. La validez de la teoría sobre la fase de transición de los lípidos, ha sido cuestionada ya que se ha observado que, en las plantas susceptibles a los daños por frío, solamente entre el 2-5% de los lípidos sufren la fase de transición a temperaturas de daño por frío (Raison y Wright, 1983; Raison y Orr, 1986), por lo que no parece claro que un cambio en una pequeña fracción de lípidos pueda influir tanto en el desarrollo de los daños (Salveit y Morris, 1990). Otro aspecto problemático de esta teoría es que la temperatura a la cual tiene lugar la fase de transición en la membrana depende del grado de insaturación de los ácidos grasos de los lípidos. Además, no siempre existen diferencias en la composición de los lípidos de la membrana entre las especies sensibles y tolerantes (Patterson et al., 1978;

Priestley y Leopold, 1979). El mutante de *Arabidopsis thaliana* fab1, tolerante a las bajas temperaturas, contiene un elevado nivel de ácidos grasos saturados en fosfatidilglicerol, muy superior al que presentan especies sensibles al frío (Wu y Browse, 1995).

Aunque el "sensor" de las plantas a las bajas temperaturas no ha sido identificado definitivamente, los descubrimientos de los últimos años continúan apuntando, entre otros factores, hacia una disminución en la fluidez de la membrana (Beck et al., 2007). La cianobacteria *Synechocystis* PCC6803 tiene al menos dos sensores para bajas temperaturas, uno de los cuales es una histidina quinasa clásica, y posee los componentes proteicos Hik33 y Hik19 (Browse y Xin 2001). Debido a que no se han detectado homólogos a los genes *Hik33* y *Hik19* en plantas, se han propuesto conceptos alternativos a la percepción del frío. Uno de ellos sugiere que el aumento de la viscosidad de membrana afecta a los canales de Ca^{2+}, induciendo un aumento de la concentración de calcio citosólico, el cual sucesivamente activa una MAPK u otras cascadas de transducción de señales (Monroy y Dhindsa, 1995) (Fig. I).

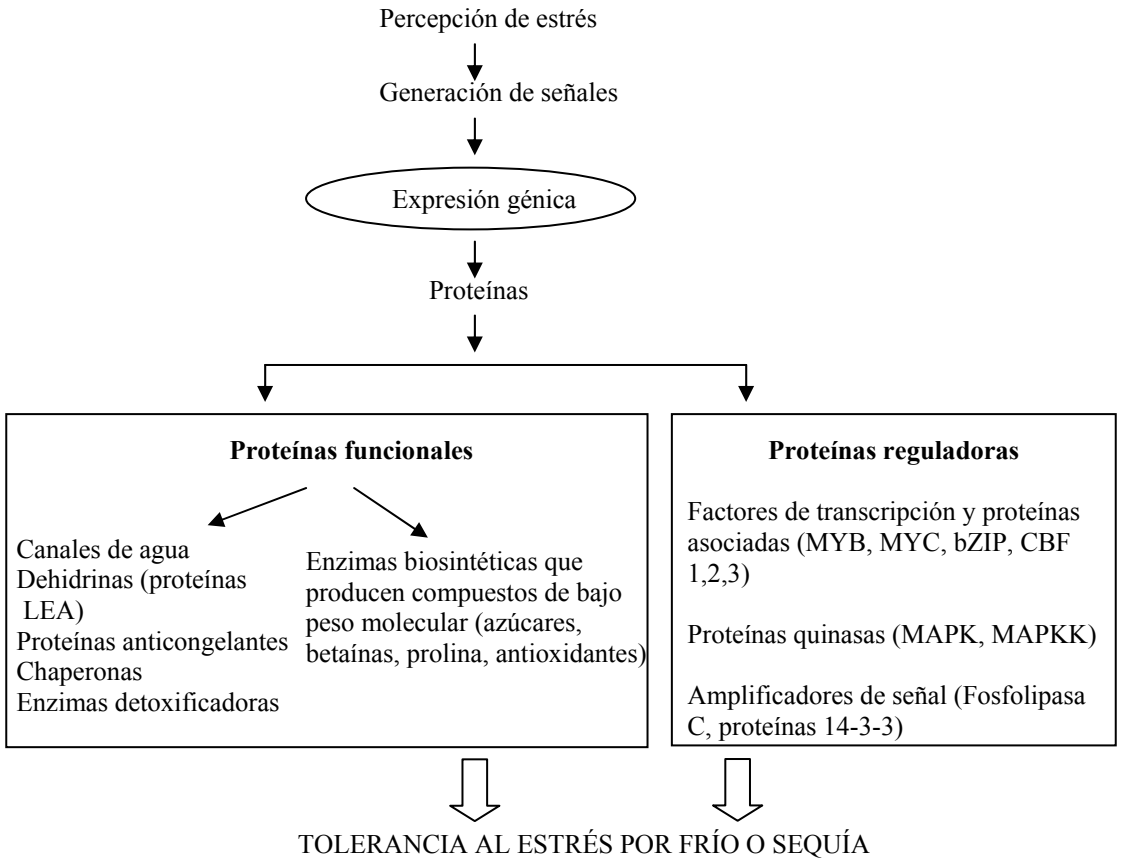


Fig. I. Factores de tolerancia a estrés producidos por las plantas en las respuestas adaptativas a bajas temperaturas y sequía.

A) Estrés oxidativo

Los estreses abióticos activan múltiples reacciones de defensa en las plantas, algunas de las cuales pueden proteger frente a distintos estreses. Las enzimas catalasa (CAT), superoxido dismutasa (SOD), ascorbato peroxidasa (APX), peroxidasa (POD) y glutation reductasa (GR), pueden proteger frente al daño causado por radicales libres o ROS (como son el superóxido, el oxígeno singlete y el radical hidroxilo) generados por distintos estreses incluyendo las altas y bajas temperaturas (Wang, 1995). El almacenamiento a bajas temperaturas puede alterar el equilibrio entre la producción de radicales libres y los mecanismos de defensa a favor del primero. Así, el balance entre la formación y la detoxificación de ROS es crítico para la supervivencia de la célula durante

el almacenamiento en frío (Zhang et al., 1995). El complejo sistema antioxidante por el cual las plantas se protegen contra los efectos de las especies reactivas de oxígeno incluye, a parte de las enzimas mencionadas anteriormente, antioxidantes liposolubles (α-tocoferol y β-caroteno), reductores hidrosolubles (ascorbato y glutation) (Zhang et al., 1995) y compuestos derivados de la ruta de los fenilpropanoides (fenoles, antocianos y estilbenos entre otros).

Las plantas que contienen niveles más elevados de antioxidantes, enzimáticos o no enzimáticos, son más tolerantes a las bajas temperaturas y otros tipos de estrés. La transformación de plantas de tabaco con el gen de la *Cu/Zn-SOD* de guisante indujo un aumento en la resistencia al estrés oxidativo causado por la exposición a las bajas temperaturas (3ºC) en presencia de luz de intensidad moderada (Gupta et al., 1993). También se sabe que las plantas modulan sus defensas antioxidantes cuando se exponen a bajas temperaturas. Se ha observado un aumento en la actividad APX y POD en pimiento dulce (Yamauchi et al., 1975), y de SOD y CAT en tomate en respuesta al frío (Michalski y Kaniuga, 1981). En las variedades de manzana Golden Delicious y Fuji almacenadas en frío la actividad CAT se duplicó; mientras que la SOD disminuyó en Golden Delicious y aumentó en Fuji (Masia 1998).

En uva, los estudios realizados con enzimas antioxidantes están relacionados con la infección por distintos patógenos (Musetti et al., 2007). Hasta el momento no se ha estudiado la relación de estas enzimas con las bajas temperaturas.

B) Regulación proteica

Son numerosos los cambios fisiológicos y bioquímicos en frutos inducidos por la exposición a bajas temperaturas (Wang, 1990). Estos cambios podrían estar relacionados con modificaciones en la estructura terciaria de proteínas específicas (Linderstrom-Lang y

Schellman, 1959), con cambios en su actividad enzimática y en la población de mRNAs específicos (Watkins et al., 1990).

Con respecto al metabolismo de los fenilpropanoides, enzimas clave como la PAL y la chalcona sintasa (CHS) presentan cambios en su actividad (Sanchez-Ballesta et al., 2000; Wang et al., 2007) y en sus características cinéticas (Maldonado et al., 2007) como respuesta a las bajas temperaturas en diversos frutos. Como producto final de la ruta catalizada por la enzima PAL, se originan compuestos relacionados con la necrogénesis y con el pardeamiento de los productos vegetales (Dangyang y Salveit, 1989), así como otros compuestos implicados en la coloración, textura y capacidad antioxidante de los mismos. La inducción de la PAL se ha considerado un mecanismo de defensa frente distintos estreses bióticos y abióticos (Haga et al., 1988). En cítricos, se ha observado que el flavedo era capaz de responder al ataque por patógeno aumentando los niveles de transcrito de la *PAL* así como la actividad de la misma enzima (Ballester et al., 2006).

Las bajas temperaturas también pueden inducir cambios en enzimas hidrolíticas de la pared celular. El almacenamiento a bajas temperaturas (7ºC) retrasó la maduración del aguacate (*Persea americana* Mill cv. Hass) y retardó la acumulación de mRNAs que codifican enzimas relacionadas con el metabolismo de la pared celular como celulasa, poligalacturonasa y enzima formadora de etileno (Dopico et al., 1993). Asimismo, se han descrito cambios en las actividades de las enzimas pectinolíticas en melocotones almacenados a bajas temperaturas, observándose un incremento en la actividad de la pectinesterasa así como una inhibición de la actividad poligalacturonasa (Ben-Arie y Sonego, 1980). La pectinesterasa es muy estable a bajas temperaturas y, en nectarinas conservadas en aire a 0ºC, su actividad se mantuvo en un nivel basal de manera que el grado de esterificación de las pectinas continuamente disminuyó durante el almacenamiento (Lurie et al., 1994); mientras que la actividad de la poligalacturonasa fue

baja en estas condiciones. Estos cambios en las actividades de las enzimas hidrolíticas de pared podrían explicar parte de los cambios en la textura de los frutos durante su conservación a bajas temperaturas (Zhou et al., 2000).

C) Regulación Hormonal

C.1) Etileno

El incremento en etileno es una respuesta común de las plantas frente a distintos tipos de estrés, tales como las bajas temperaturas, sequía, heridas, irradiaciones, ozono y el ataque por patógenos (Yang y Hoffman, 1984). El hecho de que algunas de estas respuestas puedan ser inducidas por la aplicación exógena de etileno, sugiere que esta hormona pueda actuar como una señal que coordina las distintas respuestas de las plantas ante situaciones adversas (Wang et al., 1990). Se ha observado una estimulación considerable en la síntesis de etileno en frutos almacenados entre 0 y 5ºC tales como melón (Lipton y Wang, 1987), tomate (Lurie y Klein, 1991), pera (Blankenship y Richardson, 1985) y mandarina (Zacarias et al., 2003).

En uva, Chervin et al. (2004) estudiaron la relación entre el etileno, el desarrollo y la maduración de los frutos, observando que, aunque la uva está clasificada como un fruto no climatérico, presenta un incremento transitorio de producción endógena de etileno justo antes de la averización. Por otro lado, Grimplet et al. (2007) analizaron la expresión de mRNAs que codifican proteínas implicadas en el metabolismo del etileno en piel de uva procedente de vides con déficit de agua y observaron una sobreexpresión significativa de los transcritos. Sin embargo, no existen, hasta el momento, datos en cuanto al papel de esta hormona en uvas almacenadas a bajas temperaturas.

C.2) Ácido abcísico y ácido giberélico

La exposición de los vegetales a bajas temperaturas puede afectar al estado hídrico de los mismos y, en consecuencia, a la producción de ABA (Walton, 1980), pero también puede afectar directamente a los niveles de esta hormona (Daie et al., 1981). El estrés por sequía incrementa la producción de ABA y produce el cierre de estomas e induce la expresión de genes asociados a estrés por sequía (Seki et al., 2007).

Se ha observado que entre las respuestas de las plantas para habituarse o aclimatarse al frío está el aumento del contenido de ABA y que este incremento es mayor en los tejidos que toleran mejor las bajas temperaturas (Ryu y Li, 1994). Asimismo, la aplicación exógena de ABA incrementa la tolerancia a las bajas temperaturas en distintas especies vegetales (Wang, 1990). El ABA endógeno producido por manzanas almacenadas a bajas temperaturas podría estar asociado con la tolerancia de las mismas al estrés por bajas temperaturas (Yoshikawa et al., 2007). Sin embargo, en mandarinas Fortune almacenadas en frío, se ha observado que los niveles de ABA pueden incluso disminuir tanto en las variedades tolerantes como en las variedades sensibles al frío (Lafuente et al., 1997; Lafuente y Sala, 2002).

Además de los reguladores mencionados anteriormente, el aumento en la tolerancia a las bajas temperaturas se ha asociado con variaciones en ácido giberélico. En uva de mesa almacenada en frío y tratada con 100 ppm de ácido giberélico, se ha observado que los frutos mantuvieron una mayor integridad de la membrana y que se redujo notablemente la caída de fruto, el pardeamiento del raquis y la podredumbre. Además, se observó una disminución en el contenido de sólidos solubles y vitamina C, así como una mejoría en la firmeza de la uva respecto a los frutos control (Deng et al., 2006).

D) Mecanismos moleculares

Se han identificado un gran número de genes regulados por las bajas temperaturas, algunos con una posible función reguladora y otros de función desconocida hasta el momento (Lee et al., 2005). Sin embargo, debido a que la mayoría de los estudios realizados se han hecho a nivel de planta, se sabe poco de los cambios moleculares que ocurren en los frutos durante su almacenamiento a bajas temperaturas.

Estudios comparativos han podido relacionar el aumento en los niveles de expresión de mRNAs homólogos a genes inducidos por las bajas temperaturas con las diferencias en tolerancia al frío de algunos cultivares. Estos incluyen genes *COR* (del inglés *cold-regulated*), que codifican un conjunto de pequeñas proteínas hidrofílicas denominadas dehidrinas o LEAs (del inglés *late embryogenesis abundant*) (Close, 1997); genes implicados en la biosíntesis de proteínas con función crioprotectora y/o anticongelante (AFPs, del inglés *Antifreeze Proteins*) (Hon et al., 1994) y genes relacionados con la defensa de las plantas frente al estrés oxidativo (Sung et al., 2003). Además se ha identificado y caracterizado un activador transcripcional de elementos promotores de varios genes *COR* (*CBF1*) (Jaglo-Ottosen et al., 1998) y genes implicados en la desaturación de ácidos grasos, relacionados con la adquisición de la tolerancia al frío en plantas de tabaco (Kodama et al., 1995).

Las LEA fueron las primeras proteínas relacionadas con la respuesta a la deshidratación en plantas (Baker et al., 1988). Desde entonces, se ha descrito en numerosos tejidos vegetales la posible relación entre la acumulación de este tipo de proteínas y la aclimatación de las plantas al estrés por frío (Bray 1993). La expresión de genes que codifican proteínas del tipo *dehidrina* (grupo 2 de las LEA), a menudo se induce en los tejidos vegetales como respuesta a la deshidratación, al estrés por bajas temperaturas o a la aplicación de ABA. Por ello se ha sugerido que podrían interactuar y estabilizar

membranas y macromoléculas, previniendo daños estructurales y manteniendo la actividad de enzimas esenciales (Svensson et al., 2002). Aunque las dehidrinas están ampliamente distribuidas y se han encontrado en plantas vasculares, helechos, musgos, líquenes y algas, sus funciones moleculares no son bien comprendidas ya que no catalizan ninguna reacción metabólica (Beck et al., 2007). Dehidrinas de cítricos (Hara et al., 2001; Sanchez-Ballesta et al., 2004), melocotón (Winiewski et al., 1999), trigo (Houde at al., 1995), cebada (Bravo et al., 2003) y espinaca (Kazuoka y Oeda, 1994) presentan actividad crioprotectora, así como actividad anticongelante en el caso de la dehidrina de melocotón (Winiewski et al., 1999). En hojas de *Vitis riparia* y *Vitis vinifera*, la deshidratación, las bajas temperaturas y el tratamiento con ABA indujeron la expresión de dos dehidrinas (Xiao y Nassuth, 2006).

Estudios realizados *in vitro* con cereales tolerantes a las bajas temperaturas como centeno, trigo y cebada han demostrado una acumulación de proteínas AFPs (Antikainen y Griffith, 1997). Este tipo de proteínas presentan un porcentaje de homología muy alto con PRs, en concreto con endoquitinasas de clase I, β-1,3-glucanasas y taumatina (Hon et al., 1994).

En *Arabidopsis*, se ha observado que los factores de transcripción CBF/DREB1 son reguladores críticos de la expresión génica en la señal de transducción de aclimatación al frío. Estos factores de transcripción han sido los primeros activadores transcripcionales en los que se ha demostrado su papel controlando la expresión de genes regulados por las bajas temperaturas (Thomashow, 1999; Van Buskirk y Thomashow, 2006). Sin embargo, Haake et al., (2002) aislaron un homólogo de las proteínas CBF/DREB1 (CBF4), que juega un papel similar durante la adaptación a la sequía, pero su expresión génica no está regulada por las bajas temperaturas. En uva (*Vitis vinifera* y *Vitis riparia*) la expresión de tres genes que cofican CBFs fue baja a temperatura ambiente y aumentó durante el tratamiento con bajas temperaturas (4ºC) en hojas jóvenes, cabezas apicales, yemas y tallos

jóvenes. Los transcritos de *VvCBF1, 2* y *3* que también se acumularon en respuesta a sequía y tratamientos con ABA exógeno, podrían indicar que la uva contiene genes *CBF* únicos (Xiao et al., 2006).

Otros genes cuya expresión se induce por las bajas temperaturas codifican polipéptidos que presentan homología con proteínas transportadoras de lípidos (nsLTP) (del inglés *non-specific lipid transfer*). Experimentos con gramíneas demostraron una acumulación de estas proteínas cuando las plantas fueron expuestas a condiciones de estrés hídrico, salino o por frío (Hughes et al., 1992; White et al., 1994).

También se ha identificado una espermidina sintasa (*OsSPDS2*) implicada en la síntesis de poliaminas en raíces de plantas de arroz asociada a la respuesta a las bajas temperaturas (Kim et al., 2004). Dicha implicación se ha confirmado en plantas transgénicas de *A. thaliana* que sobreexpresaban un gen de *SPDS* y que resultaron ser más tolerantes al frío (Kasukabe at al., 2004).

E) Otros compuestos endógenos

E.1) Poliaminas

Las poliaminas son pequeños policationes que pueden participar en distintos procesos como la regulación de la replicación del DNA, transcripción de genes, división celular, el desarrollo de órganos, maduración, senescencia y en respuesta a diferentes estreses bióticos y abióticos (Bouchereau et al., 1999). Putrescina (Put), espermidina (Spd) y espermina (Spm) son las poliaminas más abundantes en bacterias, plantas y animales. La hipótesis de que la síntesis de poliaminas puede estar relacionada con la defensa de los tejidos vegetales al frío, está basada en la capacidad de estos compuestos para estabilizar las membranas celulares (Wang, 1987). Asimismo, se piensa que su modo de acción frente

al estrés y la senescencia puede ser debido a la capacidad para secuestrar radicales libres que se forman en la peroxidación de lípidos (Drolet et al., 1986).

Los niveles de las poliaminas en los tejidos vegetales varían en respuesta al estrés hídrico (Wang y Steffens, 1985), osmótico (Tiburcio et al., 1990) y a las bajas temperaturas (Wang, 1987; Escribano y Merodio, 2000). En general, durante la exposición de frutos a bajas temperaturas, disminuyen los niveles de Spd y Spm y aumentan los niveles de Put (Serrano et al., 1996), al igual que en otros tipos de estrés (Bouchereau et al., 1999). La síntesis de ciertas poliaminas endógenas se puede ver incrementada por la aplicación exógena de las mismas, así como por el empleo de algunos tratamientos de acondicionamiento, lo que aumenta la tolerancia de los productos vegetales al frío. Así, en melocotones tratados con Put y almacenados a 2ºC, se vio que aumentaba la firmeza de los frutos y se redujo la susceptibilidad del fruto a ser dañado mecánicamente (Martínez-Romero et al., 2000). En uvas, los estudios se han centrado en el análisis de las distintas poliaminas (libres, conjugadas y ligadas a pared) en órganos de la variedad Cabernet Sauvignon en diferentes estados del desarrollo. Se han observado diferencias en la distribución de las poliaminas en función de los órganos estudiados, y del estado de desarrollo en que se encontraban; pero, en general, se han encontrado niveles bajos de poliaminas en todos los órganos de la fruta en etapa de maduración (Geny et al., 1997). También se han estudiado las poliaminas como respuesta al ataque por hongo en distintos órganos (yemas florales, flores y bayas pequeñas) de vides Cabernet Sauvignon; y se ha descrito un aumento de las poliaminas ligadas a pared y una disminución de las libres en los órganos dañados (particularmente Put y diaminopropano) (Rifai et al., 2004). Hasta el momento se desconoce el papel de las poliaminas en el mecanismo de respuesta de las uvas a las bajas temperaturas.

E.2) Betaína glicina

La betaína glicina es una amina anfotérica cuaternaria que parece proteger las plantas manteniendo el balance de agua entre las células y el ambiente, y mediante la estabilización de la estructura y actividad de las macromoléculas (Sakamoto y Murata, 2002). Algunas plantas como la espinaca y la cebada, acumulan un nivel relativamente alto de betaína glicina en sus cloroplastos, mientras que otras, tales como *Arabidopsis* y tabaco, no sintetizan este compuesto (Sakamoto y Murata, 2002). Plantas de *Arabidopsis* transgénicas que expresaron genes de glicina sarcosina metiltransferasa (*ApGSMT*) y dimetilglicina metiltransferasa (*ApDMT*) procedentes de cianobacterias halotolerantes, acumularon altos niveles de betaína glicina e incrementaron su tolerancia a estreses de sequía, alta salinidad y bajas temperaturas (Waditee et al., 2005).

E.3) Ácido salicílico y ácido jasmónico

Recientemente, también se ha centrado la atención en el SA debido a su habilidad para inducir termotolerancia en plantas de mostaza (Dat et al., 1998, a,b), o proteger contra el estrés por bajas temperaturas en plantas de maíz (Janda et al., 1999), tomate (Ding et al., 2002) y trigo (Tasgin et al., 2003). El SA podría estar relacionado con el estrés oxidativo jugando un papel esencial en prever el daño oxidativo (Bowler et al., 1994). Uno de los modos de acción propuestos para el SA es la inhibición de la CAT, una de las enzimas eliminadoras de H_2O_2, de manera que las concentraciones celulares de H_2O_2 aumentarían y actuarían como mensajero secundario activando genes asociados a defensa (Chen et al., 1993). Shulaev et al. (1997) demostraron que el metil salicilato (MeSA) podría actuar como una señal aérea, la cual activaría la resistencia al daño y la expresión de genes asociados a defensa en plantas vecinas y en tejidos sanos de plantas infectadas. En manzanas almacenadas a bajas temperaturas (-2ºC), se observaron mayores

concentraciones endógenas de JA que en las manzanas control mantenidas a 20ºC (Yoshikawa et al., 2007). Esto sugiere que el JA podría estar asociado con la tolerancia de las manzanas al estrés por bajas temperaturas.

E.4) Azúcares y polialcoholes

La desecación que puede ocasionar el almacenamiento a bajas temperaturas se ha correlacionado con la acumulación de ciertos azúcares (tipo galactinol, trehalosa y fructano) y polialcoholes (como manitol y D-ononitol) (Bartels y Sunkar, 2005). Plantas de *Arabidopsis* transgénicas que sobreexpresaron *CBF3/DREB1A* fueron tolerantes a estreses de sequía y frío (Avonce et al., 2004), y acumularon más galactinol y rafinosa que las plantas de tipo salvaje (Valliyodan y Nguyen, 2006).

4. TECNOLOGÍAS PARA LA CONSERVACIÓN DE UVA DE MESA. TRATAMIENTOS GASEOSOS.

Para evitar el uso de fungicidas sintéticos y cubrir la demanda del consumidor, surge la necesidad de disponer de tratamientos alternativos no químicos que preserven la calidad postcosecha de frutos.

4.1. Generadores de dióxido de azufre

El dióxido de azufre (SO_2) se emplea en frutos mínimamente procesados para evitar procesos fermentativos, la aparición de mohos y pardeamientos (Jiang et al., 2002). El tratamiento con SO_2 y bajas temperaturas utilizado para la conservación de litchis (*Litchi chinensis* Sonn), aumentó el periodo de vida útil y disminuyó el pardeamiento de los mismos (Ray et al., 2005).

La fumigación con SO_2 es el método más comúnmente utilizado para controlar podredumbres durante la conservación en frío de racimos de uva (Harvey y Uota, 1978; Crisosto et al., 1994). Al principio, el SO_2 se aplicó a uva de mesa en altas concentraciones en exposiciones cortas de tiempo o en bajas concentraciones y exposiciones prolongadas. A mediados de los años sesenta, la exposición continua de uvas a SO_2 por largos periodos de tiempo se consiguió gracias al desarrollo de generadores químicos de fase doble (rápido y lento) (Nelson y Ahmedullah, 1972, 1976). Estos generadores pueden producir SO_2 durante varias semanas en ambientes refrigerados y con alta humedad (Lagunas-Solar et al., 1992). El almacenamiento de cultivares de uva de mesa a 2ºC con generadores de SO_2 previno la podredumbre de la uva sin tener ningún efecto sobre la acidez, sólidos solubles o color, aunque su eficacia fue variable dependiendo del cultivar (Morris et al., 1992). Sin embargo, Crisosto et al. (2002) mostraron que la concentración de SO_2 necesaria para eliminar esporas y evitar el crecimiento del micelio inducía daños en la baya y el raquis. El nivel de SO_2 por encima del cual se han observado pérdidas en la calidad de uvas de mesa es de 10 mg/kg (Crisosto y Mitchell, 2002). Concentraciones elevadas de SO_2 producen blanqueamientos en la superficie de las bayas durante su almacenamiento y son especialmente visibles en variedades de uva de mesa tintas (Winkler et al., 1974). Además, los residuos de SO_2 son dañinos para la población alérgica a sulfitos, siendo 10 μL/L el umbral máximo tolerado para estos residuos presentes en frutos según la Administración de Drogas y Alimentos de los Estados Unidos (Crisosto et al., 1994); mientras que la Unión Europea ha prohibido su uso (EU Directive95/2/CE). Por otro lado, no se puede emplear SO_2 en uvas certificadas de obtención orgánica (Gabler y Smilanick, 2001).

4.2. Atmósferas controladas

Las atmósferas controladas (CA, del inglés *Controlled Atmospheres*) contribuyen a mantener la calidad y prolongar el periodo de la conservación de productos vegetales (Beaudry, 1999). Niveles reducidos de O_2 y/o enriquecidos de CO_2 pueden reducir la respiración, retrasar la maduración, disminuir la producción de etileno, retardar el ablandamiento y ralentizar los cambios en la composición debido a la maduración, resultando todo esto en un aumento de la vida útil (Farber, 1991). Además de ser una tecnología postcosecha competitiva, el empleo de CA es una alternativa a la fumigación química (Yahia y Vazquez-Moreno, 1993). En los últimos años los progresos en su desarrollo tecnológico se han centrado en el empleo de fluidos criogénicos como el nitrógeno líquido que, además de favorecer el establecimiento de bajos niveles de O_2, actúan como medio de enfriamiento rápido (Mahajan y Goswami, 2007).

Los efectos de las CA con alto CO_2 en la calidad y fisiología de la uva han sido evaluados en distintas variedades (Artés-Hernández, et al., 2004; Crisosto et al., 2002). Estos resultados han mostrado que las CA pueden retardar la senescencia, reducir la respiración de la baya y del raquis, mantener la firmeza de la baya, disminuir el oscurecimiento del raquis y reducir, así como retrasar, la podredumbre. Sin embargo, las limitaciones del empleo del almacenamiento en CA son principalmente el metabolismo fermentativo y el estrés fisiológico que pueden dar como resultado el desarrollo de sabores y aromas anómalos, el oscurecimiento del raquis y la inducción de podredumbre por hongo después del paso a aire durante el periodo de vida útil (Grierson et al., 1966; Kader, 2002).

Métodos combinados de conservación tales como CA y generadores de SO_2 aumentaron el periodo de almacenamiento de los frutos, disminuyeron las pérdidas de peso, textura y color, y retrasaron tanto el ataque fúngico como el marchitamiento del

raquis (Pretel et al., 2006). Sin embargo, los niveles de ácido tartárico y cítrico mostraron un brusco incremento.

En uvas cv. Kyoho, el tratamiento con altas concentraciones de CO_2 y bajo O_2 suprimió las actividades de las enzimas celulasa, poligalacturonasa y peroxidasa; y como resultado se redujo la abscisión de las bayas de uva durante el almacenamiento (Deng et al., 2007). Las uvas cv. Thompson Seedles toleraron altos niveles de CO_2 durante más de 2 semanas a 0ºC (Ahumada et al., 1996) y se consiguió un control adecuado de la infección por *Botrytis* usando altos niveles de CO_2 (Berry y Aked, 1997; Crisosto et al., 2002,b). En este mismo cultivar y en Red Globe se estudió la eficacia de atmósferas ricas en CO_2 sobre el control de podredumbre durante el almacenamiento de la uva a 0ºC y se comparó con los generadores de SO_2. Se observó que concentraciones de 15% y superiores de CO_2 resultaron tan buenas como los generadores de SO_2 para controlar al hongo *Botrytis*; sin embargo, los tallos y pedicelos fueron más verdes cuando las uvas se almacenaron con generadores de SO_2 (Retamales et al., 2003).

4.3. Atmósferas modificadas

Las atmósferas modificadas (MAP, del inglés *packaging modified atmospheres*) pasivas o activas se presentan como una tecnología alternativa para la conservación de productos enteros o mínimamente procesados que presentan sensibilidad a distintos factores abióticos como las bajas temperaturas, altas o bajas HRs, etc. En estos casos, el almacenamiento en condiciones variables de composición gaseosa y humedad relativa de la atmósfera, permite aumentar el periodo de vida útil y mantener la calidad del producto vegetal. Generalmente, porcentajes de 3–8% CO_2 y 2–5% O_2 son recomendados para la conservación de frutas y verduras mediante MAP (Farber, 1991).

En cerezas, frutos no climatéricos altamente perecederos, se ha observado que la refrigeración de las mismas en MAP mejora ciertos parámetros de calidad del fruto (Apel et al., 1982), reduciendo las podredumbres causadas por desarrollo fúngico (De Vries-Patterson et al., 1991), inhibiendo el desarrollo del color de los frutos (Artés et al., 2001) y prolongando la vida postcosecha de los mismos (Remon et al., 2000).

El empleo de MAP se ha comprobado que mantiene la calidad organoléptica de uvas durante su almacenamiento. Sin embargo, la baja intensidad de respiración de la uva de mesa y por consiguiente las concentraciones de CO_2 alcanzadas dentro de los paquetes, no fueron lo suficientemente elevadas para actuar como fungicida (Martínez-Romero et al., 2003). Sin embargo, concentraciones de 15 kPa de O_2 y 10 kPa de CO_2 fueron eficaces para evitar el marchitamiento del raquis y el ablandamiento de los granos, así como para mantener la calidad visual, sabor y textura, controlando el desarrollo de podredumbres (Artés-Hernández et al., 2004).

Otros estudios mostraron que el empleo de MAP en combinación con ácido acético (Moyls et al., 1996), clorina (Zoffoli et al., 1999), eugenol (Valero et al., 2006) o generadores de SO_2 comercial (Artés-Hernández et al., 2004) en la conservación de uvas de mesa redujeron significativamente el daño provocado por *B. cinerea*; sin embargo, en estos estudios, el problema de los residuos persistió.

4.4. Ozono

La aplicación exógena de ozono permite el control del crecimiento y desarrollo microbiológico de los frutos así como de las instalaciones. Asimismo, retrasa la maduración de los frutos, lo que permite prolongar considerablemente el tiempo de conservación de los mismos. Además, la exposición a niveles de ozono por debajo de los críticos puede resultar en la inducción de compuestos de defensa, tales como estilbenos,

contra el estrés oxidativo (Roseman, 1991). Se ha observado que la aplicación de ozono, a lo largo de la conservación a bajas temperaturas de la uva de mesa, aumenta su contenido de fenoles totales (Artés-Hernánadez et al., 2007). Sin embargo, experimentos sobre fumigación con cantidades controladas de ozono revelaron que niveles bajos (<0.15 μL/L) afectan al crecimiento de las plantas y desarrollo, siendo los daños resultantes blanqueamientos, lesiones, clorosis y necrosis en las hojas de las plantas (Treshow, 1989). En tomate cherry, el tratamiento con altas concentraciones de ozono tuvo un efecto bactericida muy efectivo y no se observaron cambios en la textura; pero causó cambios en el color de la superficie de los mismos y podría resultar en pérdidas organolépticas y nutricionales (Das et al., 2006).

4.5. Jasmonatos y salicilatos

El JA y su ester metílico, MeJA, se han encontrado de forma natural en un amplio rango de plantas superiores. El JA es el producto final de la oxidación enzimática de ácidos grasos insaturados, linoleico y linolénico principalmente, y la enzima clave de esta ruta de los octadecanoides es la lipooxigenasa (LOX) (Vick y Zimmerman, 1984). Son fitohormonas lipídicas, que actúan como moléculas señalizadoras de la respuesta de las plantas a numerosas situaciones de estrés y participan en diversos procesos de desarrollo. La volatilidad del MeJA permite que los tratamientos sean aplicados sin necesidad de la inmersión del fruto en agua mientras que el JA, al ser más soluble en agua, es adecuado para usar en solución (Tripathi y Dubey, 2002).

Se ha demostrado que varios compuestos derivados del JA activan genes que codifican proteínas antifúngicas tales como tionina (Andresen et al., 1992), osmotina (Xu et al., 1994), y otros genes implicados en la biosíntesis de fitolalexinas (Gundlach et al., 1992). Se ha observado que el JA aumenta la tolerancia al estrés por frío de varias plantas

(Meir et al., 1996). En pimiento verde, se observó que el pretratamiento con vapores de MeSA o de MeJA indujo cambios en la expresión de genes que codifican la alternativa oxidasa (*AOX*) así como en las enzimas implicadas en la defensa frente al estrés oxidativo. El aumento de los niveles de los transcritos de *AOX* por MeJA o por MeSA se ha correlacionado con una menor incidencia de daño por frío (Fung et al., 2004).

Además, el tratamiento con vapores de MeJA redujo el porcentaje de infección causado por *B. cinerea* en fresa (Moline et al., 1997). También se observó que el tratamiento con bajas concentraciones de MeJA o de MeSA aumentaba substancialmente la resistencia de frutos de tomate al estrés por frío y disminuía la incidencia de podredumbre durante el almacenamiento a bajas temperaturas. Asimismo, dicho tratamiento indujo la síntesis de algunas proteínas asociadas a estrés, como PRs, que aumentaron la tolerancia al frío y la resistencia a los patógenos (Ding et al., 2002). En *Arabidopsis*, el pretratamiento con MeJA causó una eficiente reducción del desarrollo de infecciones fúngicas (Thomma et al., 2000).

Se ha descrito que el tratamiento exógeno con el análogo de JA, n-propil dihidrojasmonato, favorece el desarrollo del color de las bayas (Fujisawa et al., 1996). Además, recientemente se ha descrito que el tratamiento de hojas de *Vitis* con SA desencadenó el sistema de resistencia adquirida (SAR, del inglés *Systemic Acquired Resistance*) y fue efectivo para controlar la podredumbre (Aktas y Guven, 2007).

4.6. Otros tratamientos

a) Etanol y sorbatos

Etanol y sorbato potásico son conocidos aditivos de los alimentos con potente actividad antimicrobiana (Larson y Morton, 1991). Se ha descrito que los baños y vapores

en etanol son efectivos en la conservación postcosecha de melocotones, cítricos y uvas de mesa (Gabler y Smilanick, 2001; Karabulut et al., 2003). Los sorbatos son preservantes comunes de comida con muchas aplicaciones, y su espectro de actividad incluye *B. cinerea* (Sofos, 1989). Karabulut el al. (2005) analizaron el tratamiento combinado de etanol (20%) y sorbato potásico (0.5 ó 1%) en uvas de mesa cv. Flame Seedles para controlar el ataque por *B. cinerea* y observaron que la combinación de ambos compuestos tuvo igual eficacia en reducir la incidencia del hongo que los generadores comerciales de SO_2, pero sin observarse los daños en las bayas citados anteriormente para el tratamiento con SO_2.

b) Hexenal

El compuesto volátil (E)-2-Hexenal es ubicuo y fuertemente antifúngico, habiéndose demostrado su actividad contra *B. cinerea* (Fallik et al., 1998). Archbold et al. (1999) demostraron que es un fumigante eficiente en controlar el hongo de uvas de mesa apirenas. El vapor de hexenal inhibió el crecimiento de las hifas de *Penicillium expansum* y *B. cinerea* en ensayos *in vitro* y sobre rodajas de manzana (Song et al., 1996).

c) Ácido acético

La fumigación con ácido acético se ha demostrado que es adecuada para uso comercial de albaricoques y ciruelas (Liu et al., 2002), cerezas dulces (Chu et al., 2001) y uvas (Sholberg et al., 1996). El ácido acético es un intermediario del metabolismo fermentativo y se acumula en muchos frutos (Nursten, 1970). Sholberg y Gaunce (1995) mostraron que bajas concentraciones de ácido acético en aire fueron extremadamente efectivas para controlar los conidios de *B. cinerea* en manzanas sin tener efecto tóxico. Asimismo, la fumigación con ácido acético protegió del deterioro a las uvas conservadas en MAP a 0ºC hasta 2 meses, y se presentó como una forma alternativa para aumentar su

tiempo de vida útil (Moyls et al., 1996). El empleo de ácido acético podría ser también una alternativa al SO_2, que se utiliza habitualmente para controlar la podredumbre de uvas de mesa.

d) Pretratamientos con CO_2

Pretratamientos con altos niveles de CO_2 sobre frutos tropicales y subtropicales durante la conservación a temperaturas inferiores a la crítica son efectivos en el control de los daños por frío (Merodio y De La Plaza 1997; Merodio et al., 1998). Cuando la aplicación de estos pretratamientos se realizó a temperatura ambiente se retrasaron los cambios asociados a la maduración y senescencia en los tejidos verdes (Merodio y De la Plaza, 1997; Escribano et al., 1997). También se observó una supresión de la síntesis basal y autocatalítica de etileno (sin modificar los bajos niveles de ACC oxidasa) y un incremento de los niveles de las poliaminas Spm y Spd (Muñoz et al., 1999).

En tomates cherry (*Lycopersicon esculentum* Mill.) conservados con altas concentraciones de CO_2 (20%) durante 3 días, y posteriormente transferidos a aire, se observó una inhibición de los cambios de color asociados a la maduración, una menor producción de etileno, así como una reducción del contenido de proteínas. También se observó una fuerte inducción de genes que codifican proteínas asociadas a estrés como una proteína de choque térmico (HSPs, del inglés *Heat Shock Proteins*) y una glutamato descarboxilasa (Rothan et al., 1997).

Por su carácter fungistático, el empleo de altas concentraciones de CO_2, se presenta como una alternativa al uso de SO_2 y de productos fitosanitarios. En este trabajo se estudia el efecto de un pretratamiento de 3 días de duración con altas concentraciones (20%) de CO_2 en la conservación de uva de mesa cv. Cardinal a bajas temperaturas. Se analizarán

distintos parámetros de calidad así como los mecanismos de respuesta de la uva asociados a la ruta de los fenilpropanoides y a las PRs durante estas condiciones de conservación.

Objetivos

El objetivo general de este trabajo es analizar las bases fisiológicas y moleculares implicadas en los mecanismos de respuesta de la uva de mesa cv. Cardinal al pretratamiento con 20% de CO_2 durante su conservación a 0ºC.

Este objetivo general se ha desarrollado según los siguientes objetivos parciales:

1. Análisis de la efectividad del tratamiento con altos niveles de CO_2 para mantener la calidad de los diferentes tejidos del racimo de uva de mesa.

2. Análisis de los marcadores moleculares de la fase inicial de conservación a 0ºC en la piel de uva de mesa y, de los principales mecanismos de protección inducidos por el tratamiento con altos niveles de CO_2 relacionados con el metabolismo de los fenilpropanoides.

3. Estudio de la eficacia del tratamiento combinado de bajas temperaturas y alto CO_2 en el control del ataque fúngico, y de la implicación de las proteínas relacionadas con la patogénesis. Estudio de la funcionalidad de una quitinasa y una β-1,3-glucanasa de clase I.

Capítulo 1

Efecto de elevadas concentraciones de CO_2 en los cambios metabólicos asociados con la respuesta de uva de mesa a 0ºC. Metabolismo de fenilpropanoides.

Los compuestos fenólicos, productos secundarios del metabolismo de las plantas, contienen uno o más anillos aromáticos y, al menos, un sustituyente hidroxilo (Dixon, 2001). Constituyen un grupo heterogéneo de productos con más de 10000 compuestos, y esta diversidad estructural se debe a la gran variedad de modificaciones en regiones específicas, que incluyen hidroxilaciones, glicosilaciones, acilaciones, sulfataciones o metilaciones (Fridman y Pichersky, 2005). Por otro lado, se ha descrito que la concentración de los compuestos fenólicos en los frutos depende de factores genéticos, ambientales y culturales (Haselgrove et al., 2000).

El interés por estos compuestos se centra en su efecto beneficioso para la salud, debido en general a sus propiedades antioxidantes, lo que resulta en un efecto antiinflamatorio y un gran potencial en la prevención del cáncer y enfermedades cardiovasculares (Hertog et al., 1992). Diversos estudios epidemiológicos indican que el consumo de altas dosis de frutas y verduras aporta beneficios para la salud en la prevención de enfermedades crónicas (Arai et al., 2000). También se ha demostrado que los fenoles juegan un papel importante en la defensa de las plantas frente a distintos estreses bióticos y abióticos. Se ha descrito un aumento de antocianos y flavonas en respuesta a altos niveles de luz visible, y se piensa que éstos compuestos ayudan a atenuar la cantidad de luz que alcanzan las células fotosintéticas (Beggs et al., 1987). También la irradiación UV provoca un aumento de flavonoides (particularmente derivados de dihidrokaempferol) y esteres de sinapato en *Arabidopsis*, así como de isoflavonoides en otras especies (Li et al., 1993; Lois, 1994). Algunos compuestos como las ligninas participan en la reparación de heridas y en el refuerzo de la pared celular, los flavonoides y/o las cumarinas protegen a las plantas del estrés oxidativo, y algunos compuestos fenólicos actúan como agentes antimicrobianos o antifúngicos (Dixon y Pavía, 1995; Ndong et al., 2003).

En los últimos años se ha prestado especial atención a los compuestos fenólicos por su implicación en la calidad de los productos hortofrutícolas (Bedgoog et al., 2005; Kalt, 2005) debido a la importancia que tienen en su aspecto y características organolépticas (Tomas-Barberán y Espin, 2001). Forman parte de esencias y pigmentos que confieren aromas, colores y sabores a los frutos; pero su degradación oxidativa catalizada por enzimas como polifenol oxidasa (PPO) y peroxidasa (POD) (Vamosvigyazo, 1981; Lei et al., 2004), es uno de los principales problemas de la industria alimentaria debido a que ocasionan el pardeamiento enzimático de frutos y vegetales frescos. La composición fenólica de los frutos y vegetales también puede ser afectada por las tecnologías de procesado de alimentos tales como tratamientos térmicos, presión, tratamientos enzimáticos, fermentación, etc. Además, procesados domésticos como cocinar (Price et al., 1998) y exprimir manualmente los frutos para obtener zumos (Häkkinen et al., 2000; Gil-Izquierdo et al., 2001) también afectan a su composición. Se ha descrito que la composición fenólica de la uva podría variar durante las diferentes etapas de desarrollo (Jordao et al., 2001), lo que podría ser atribuido a una serie de alteraciones químicas y enzimáticas de algunos fenoles durante la fase de maduración, que incluye la hidrólisis de glicósidos por glicosidasas, oxidación de fenoles por fenoloxidasas y polimerización de fenoles libres (Ryan et al., 2002).

1. Biosíntesis de los compuestos fenólicos

Aunque los compuestos fenólicos son un grupo heterogéneo desde el punto de vista metabólico, en su biosíntesis están involucradas dos vías básicas: la vía del ácido siquímico y/o la vía del ácido malónico (o de los poliacetatos).

La ruta del ácido siquímico, la principal en plantas superiores, es dependiente de luz y se inicia en los plastos por condensación de dos productos típicamente fotosintéticos,

la eritrosa 4-fosfato con el fosfoenolpiruvato (PEP), y por diversas modificaciones se obtiene el ácido siquímico, del cual derivan directamente algunos fenoles en los vegetales. La incorporación de una segunda molécula de PEP conduce a la formación de fenilalanina que, por acción de la enzima L-fenilalanina amonio-liasa (PAL), se transforma en el ácido *trans*-cinámico, sustrato común de la biosíntesis de distintos fenilpropanoides tales como antocianos, flavonoides, furanocumarinas antimicrobianas, fitoalexinas, ligninas y esteres fenólicos (Hahlbroock y Scheel, 1989; Dixon y Pavia, 1995). La ruta del ácido malónico, minoritaria en plantas superiores, da lugar a la síntesis de quinonas, xantonas y orcinoles, entre otros compuestos (Dixon y Pavía, 1995). Por otra parte, algunos de los compuestos citados anteriormente pueden originarse a través de rutas mixtas que combinan la ruta del siquimato y del malonato, como es el caso de los flavonoides (Dewick, 1994).

1.1. Enzimas reguladoras de la ruta de los fenilpropanoides: L-fenilalanina amonio-liasa, chalcona sintasa y estilbeno sintasa.

La enzima PAL cataliza la primera etapa en la ruta de los fenilpropanoides, que da lugar a una variedad de compuestos fenólicos con funciones relacionadas con estructura y defensa, que incluyen antocianos, pigmentos flavonoides, furanocumarinas, fitoalexinas, lignina y ésteres fenólicos (Hahlbrock y Scheel, 1989; Dixon y Pavía, 1995). Es la enzima clave del metabolismo de los fenilpropanoides y está regulada por distintos factores, tales como la luz, y depende además de la concentración de diferentes hormonas vegetales. En general, la actividad PAL aumenta cuando las plantas son sometidas a situaciones de estrés, como la falta de agua, infecciones fúngicas o bacterianas y radiaciones UV (Ohl et al., 1990). También se ha observado aumentos en la actividad de la PAL y acumulación de fenilpropanoides en plantas como respuesta a bajas temperaturas (Chalker-Scott et al., 1989; Leyva et al. 1995; Solecka et al. 1999; Janas et al., 2000; Sanchez-Ballesta et al.,

2000; Janas et al., 2002). Por otro lado, la manipulación de los frutos y vegetales durante la cosecha, el almacenamiento y el transporte postcosecha, pueden tener un importante impacto sobre los compuestos fenólicos y enzimas involucradas en el metabolismo fenólico, llevando a cambios (generalmente una disminución) en la calidad de los mismos (Tomás-Barberán y Espín, 2001).

En la mayoría de las especies estudiadas, la PAL está codificada por pequeñas familias multigénicas (Cramer et al., 1989) que se expresan diferencialmente en los tejidos de las plantas dependiendo de la respuesta a distintas condiciones de estrés (Lois et al., 1989). El gen que codifica la PAL así como otros genes de la biosíntesis de antocianos, han sido considerados como *cor*, y han sido propuestos como candidatos para caracterizar las respuestas moleculares de las plantas a las bajas temperaturas (Christie et al., 1994). Además de la regulación transcripcional, el estrés por frío puede también afectar a los niveles de los mensajeros de la PAL así como a su actividad enzimática (Graham y Patterson, 1982; Sanchez-Ballesta et al., 2000; LoPiero et al., 2005); aunque, en general, el nivel basal de transcritos de *PAL* y su actividad son bajos (Lee et al., 1994).

Estudios de la expresión de genes relacionados con la biosíntesis de antocianos en la piel de uva muestran que la ruta de formación de estos antocianos ocurre en dos fases. La mayoría de los genes que codifican las enzimas de la ruta (incluida la PAL) son expresados tempranamente en el desarrollo de la uva y 'de novo' después de la maduración, cuando se desarrolla el color. Además, no se ha detectado expresión de la *PAL* en la pulpa de uva en ninguna etapa del desarrollo (Boss et al., 1996).

La activación del metabolismo de fenilpropanoides podría jugar un papel importante en el desarrollo de barreras protectoras en las células dañadas por estrés. Así, plantas transgénicas con niveles de *PAL* suprimidos exhibieron necrosis espontánea (Tamagnone et al., 1998) o desarrollo más rápido y extenso de la lesión que las plantas

salvajes después de la infección por patógeno (Maher et al., 1994) sugiriendo que la inhibición de la biosíntesis de los fenilpropanoides podría comprometer el estado fitosanitario de la planta.

Un gran número de fenilpropanoides inducidos por estrés derivan del esqueleto flavonoide C15, el cual es sintetizado via chalcona sintasa (CHS) que cataliza la reacción de condensación de p-cumaril-coenzima A (CoA) y tres moléculas de malonil-CoA (Harbone y Grayer, 1988). La CHS es la enzima que cataliza el primer paso de la biosíntesis de los flavonoides. En la mayoría de las plantas, el producto inicial de la CHS es una tetrahidrochalcona que es posteriormente convertida a otras clases de flavonoides, tales como flavonas, flavanonas, flavanoles, antocianos (Holton y Cornis 1995) y 3-deoxiantocianidinas.

En estudios con uvas irradiadas con UV se ha observado que la expresión de mRNAs de CHS disminuyó en la piel de uva antes de la averización y aumentó después de la misma (Takayanagi et al., 2004). Aunque la actividad CHS es principalmente inducida por estímulos de luz (Schmelzer et al., 1988) y elicitores bióticos (Dixon y Pavía, 1995), se ha correlacionado con los mecanismos de defensa tras el estrés con ozono (Rosemann et al., 1991). Sin embargo, en líneas celulares procedentes de hojas de *Vitis* sometidas a tratamientos con ozono se observó actividad PAL mientras que no se encontraron evidencias de la implicación de la CHS en la respuesta a este gas (Sgarbi et al., 2003). Por otro lado, se han observado aumentos en los niveles de expresión de la PAL y CHS en respuesta a la infección por *Fusarium thapsinum* y *Curvularia lunata* en tejidos florales de sorgo (*Sorghum bicolor*) (Little y Magill, 2003). La acumulación coordinada de ambos mensajeros sugiere que las respuestas a los diversos estímulos bióticos y abióticos podrían ser reguladas a través de un mecanismo de control común (Loake et al., 1991).

La estilbeno sintasa (STS) es la enzima que controla la síntesis de fitoalexinas estilbénicas catalizando la última etapa de la biosíntesis de la ruta del resveratrol. La STS está codificada por una familia multigénica caracterizada por tener secuencias de nucleótidos con alta homología (Wiese et al., 1994); y es una enzima muy relacionada con la CHS ya que ambas enzimas son homodímeros que catalizan reacciones de condensación de p-cumaril-CoA con tres malonil-CoA pero con diferentes reacciones de ciclación.

Se ha descrito la expresión constitutiva de genes que codifican STS en uva (Sparvioli et al., 1994), pero esta enzima también puede ser inducida por factores abióticos como radiaciones UV, ozono o estreses bióticos (Dercks y Creasy, 1989; Adrian et al., 2000; Grimming et al., 2002). Así, la enzima STS es reconocida como el biomarcador más importante y susceptible inducido por ozono en vid y pino (Schubert et al., 1997). Además, la STS es una enzima de defensa frente al ataque fúngico. En *Vitis*, se han estudiado la STS y la PAL en respuesta a la infección por *B. cinerea* y se ha observado un incremento en la expresión de los genes tras la inoculación de las hojas con el hongo (Bézier et al., 2002).

1.2. Derivados fenilpropanoides: *trans*- resveratrol y antocianos

Los derivados fenilpropanoides son productos que tienen un papel específico en la respuesta frente a patógenos, como pantalla ultravioleta, mediante su capacidad antioxidante y de disipación de energía, así como componentes estructurales de la pared celular (Grace y Logan 2000). En uva los más destacados son los antocianos y el *trans*-resveratrol, pero también se deben mencionar las ligninas y suberinas responsables del engrosamiento de la pared celular como mecanismo de defensa.

La lignina y la suberina son polímeros complejos formados a partir de una mezcla de fenilpropanoides simples y su composición varía entre las distintas especies (Lewis y Yamamoto, 1990; Bernards y Lewis, 1992; Dixon y Pavía 1995). Estos compuestos

participan en el refuerzo de la pared celular haciendo así a las plantas más tolerantes frente a situaciones adversas; y también colaboran en los procesos de reparación de heridas contribuyendo, probablemente, a frenar el desarrollo de daños incipientes (Bernards y Lewis, 1992; Whetten y Sederoff, 1995).

Las fitoalexinas son un grupo de moléculas de estructura diversa, generalmente lipofílicas, no específicas en su actividad antifúngica y no muy potentes (Grayer y Harbone, 1994; Smith, 1996). El mecanismo de acción antimicrobiana de las fitoalexinas no se conoce bien todavía. La base de su toxicidad parece estar relacionada con la disrupción de membranas, aunque varios procesos fisiológicos y bioquímicos distintos a los cambios de permeabilidad de la membrana podrían también estar afectados (Rogers et al., 1996; Smith 1996). Las fitoalexinas no están presentes en tejidos de plantas sanas y son sintetizados a partir de precursores remotos en respuesta al ataque por patógenos o estrés, probablemente, como resultado de la síntesis de enzimas 'de novo' (Lamb et al., 1989).

En uva, la producción de fitoalexinas tales como estilbenos es una de las rutas de defensa más importantes (Langcake y Pryce, 1977). Las fitoalexinas de las especies de *Vitis* están formadas por un grupo restringido de moléculas que pertenecen a la familia del estilbeno y derivan principalmente del *trans*-resveratrol (3,5,4'-trihidroxiestilbeno). Este compuesto ha recibido últimamente mucha atención por su implicación en la salud humana (Bertelli et al., 1995; Jang et al., 1997; Hung et al., 2000); y se encuentra en las hojas de la vid y principalmente en la piel de las uvas. La producción de resveratrol depende de muchos factores y la concentración del mismo es muy dependiente del cultivar. Se acumula en respuesta a infección por hongo, radiación UV, o sustancias químicas (Langcake, 1981; Liswidowati et al., 1991; Jeandet et al., 1995; Adrian et al., 1997). Específicamente, ha sido estudiada la producción de *trans*-resveratrol en uvas y vino en respuesta a la irradiación con UV-C (Creasy y Coffee, 1988; Cantos et al., 2002). Además,

también se ha visto una buena correlación entre la producción de resveratrol y la resistencia al hongo gris (Sbaghi et al., 1995) y también al hongo negro causado por *Rhizopus stolonifer* en distintas variedades de uva (Sarig et al., 1997). El *trans*-resveratrol es sintetizado por la condensación de una molécula de p-cumaril-CoA con tres unidades de malonil-CoA en una reacción catalizada por la enzima STS. Estudios recientes muestran que el *trans*-resveratrol es fungitóxico a concentraciones fisiológicas contra *B. cinerea* (Adrian et al., 1998). Sin embargo, también se ha probado que aumenta la resistencia de las vides a otros patógenos como *Plasmopara viticola* (Dai et al., 1995), *Phomopsis viticola* (Hoos y Blaich, 1990) o *Rhizopus stonifer* (Sarig et al., 1997). Este carácter antifúngico bastante inespecífico y la acumulación selectiva del *trans*-resveratrol en la piel de la uva, lo hacen un buen candidato como pesticida natural contra el ataque de patógenos ya que aumenta la resistencia natural de las uvas a la infección fúngica. Además, debido a sus propiedades antioxidantes, el *trans*-resveratrol puede también tener efectos positivos en la conservación de los frutos durante su almacenamiento. Como consecuencia de esto, tanto el aumento de la producción endógena como la aplicación exógena del mismo podrían ser explotados para reducir los daños de la uva (Montero et al., 2003).

Los antocianos son flavonoides glicosilados biosintetizados via siquimato (Mol et al., 1989), y como miembros del grupo flavonoide, comparten la estructura $C_6C_3C_6$. Son compuestos particulares entre los flavonoides debido a su habilidad para absorber luz visible, lo que resulta en su apariencia coloreada (Jackman y Smith, 1996). El término antociano (o antocianina) se refiere a las formas glicosiladas de manera natural de estos pigmentos; y sus agliconas son designados antocianidinas. La estructura esquelética de las antocianidinas es la 2-fenilbenzopirilo o catión flavilo, el cual es polihidroxilado y puede ser polimetoxilado (Jackman y Smith, 1996). Excepcionalmente, el catión flavilo lleva grupos hidroxilo en la posición 3, 5, 7, 4` (Brouillard, 1982). Hay 18 estructuras de

antocianidinas, de las cuales, las seis estructuras más comunes son: pelargonidina, cianidina, delfinidina, peonidina, petunidina y malvidina (Strack y Wray, 1994). Sin embargo, las antocianidinas están en pocas ocasiones en su forma libre en la naturaleza. En general, la glicosilación está siempre presente en la posición 3 del anillo C y confiere solubilidad y estabilidad a los antocianos (Brouillard, 1983). La fracción de azúcar ligado a las agliconas es principalmente glucosa, pero también pueden encontrarse otros monosacáridos como ramnosa, galactosa, xylosa, arabinosa o fructosa; disacáridos como rutinosa o soforosa y trisacáridos (Jackman y Smith, 1996).

La glicosilación imparte mayor solubilidad a las moléculas en agua (Mazza y Maniati, 1993) y tiene un fuerte impacto en el color. Los 3-glicósidos son, por ejemplo, más fuertemente coloreados que los 3,5- y 5-glicósidos, cuando se comparan sus densidades ópticas (Mazza y Maniati, 1993). Con respecto a esto, el color es más o menos independiente del tipo y número de azúcares en el C-3, mientras que una glicosilación adicional en el C-5 induce un ligero cambio hacia rojo-morado (Stintzing y Carle, 2004).

La distribución de los distintos antocianos es un rasgo característico de tejidos de frutos y otras plantas. Como otros compuestos fenólicos, se encuentran localizados en la vacuola celular (Harbone, 1967; Mazza y Miniati, 1993). Los antocianos son responsables del color rojo, violeta y azul de frutas y flores (Harbone y Grayer, 1988). Aunque se acumulan esencialmente en las células epidérmicas de flores y frutos, sus principales funciones parecen ser atraer insectos y pájaros para la polinización de las flores y de animales para la diseminación de las semillas de los frutos (Timberlake y Bridle, 1982). Además, los antocianos pueden ser factores importantes junto con otros flavonoides en la resistencia de las plantas frente al ataque de insectos (Harbone y Grayer, 1988). Por ejemplo, se ha descrito que la cianidina-3-glucósido era capaz de proteger hojas de algodón contra el gusano del tabaco (Hedin et al., 1983). Además, debido a su conocida capacidad

antioxidante, se han publicado distintos trabajos donde se describen ciertos beneficios terapéuticos asociados a los antocianos como propiedades antiinflamatorias y vasoprotectoras (Lietti et al., 1976; García-Alonso et al., 2004; Oak et al., 2006).

En uva, la biosíntesis de antocianos comienza con la maduración de la baya (averización) y normalmente continúa a través de la fase de crecimiento de la misma. Es un proceso controlado genéticamente, y se sabe que prácticas tales como el uso de fertilizantes, grado de carga del cultivo, grado de poda, así como la temperatura e intensidad de la luz (Kliewer 1970), afectan mucho a la expresión de enzimas de la ruta de flavonoides, induciendo la acumulación de antocianos (Dokoozlian et al., 1995). Además, se ha visto que las bajas temperaturas aumentan significativamente los niveles de antocianos en distintas especies de plantas (Faragher 1983; Christie et al., 1994; Oren-Shamir y Levi-Nissim 1997; Stiles et al., 2007). Otros factores como variedad, región y condiciones de crecimiento, también pueden influir en los niveles de antocianos producidos y en el perfil de los diferentes pigmentos (Mazza y Maniati, 1993).

2. Regulación de los fenilpropanoides

Se ha descrito la acumulación de mRNAs de *PAL*, *STS* y *CHS* en respuesta a estreses bióticos y abióticos (Leyva et al, 1995; Brehm et al., 1999; Sanchez-Ballesta et al., 2000; Versari et al., 2001). El almacenamiento a bajas temperaturas puede tener efectos positivos o negativos en los niveles de fenoles de los productos vegetales dependiendo del producto que se trate y de la temperatura de almacenamiento. En frutos rojos como fresas, arándanos, uvas y granadas, las bajas temperaturas producen un incremento del contenido de antocianos y de derivados del ácido hidroxicinámico que favorece el color del producto (Kalt y McDonald, 1996; Gil et al., 1997; Holcroft et al., 1998; Cantos et al, 2000). Sin embargo en productos sensibles al frío como la banana, la piña, el mango o el melón, el

almacenamiento a bajas temperaturas provoca la aparición de daños por frío, que coincide con un incremento en la actividad de la PPO y la POD (Ose et al., 1995; Stewart et al., 2001; Nguyen et al., 2003; Vela et al., 2003).

También los factores medioambientales y agronómicos afectan al contenido y composición de los compuestos fenólicos. En concreto, el estatus carbono-nitrógeno afecta al contenido de fenilpropanoides en plantas de tabaco (Fritz et al., 2006); y la deficiencia de boro también puede afectar sustancialmente al contenido fenólico de las plantas (Liakopoulos y Karabourniotis, 2005). Asimismo, la presencia de metales pesados (Tahlil et al., 1999), el estrés salino (Rodríguez-López et al., 2000), la deficiencia en calcio o fosfato (Juszczuk et al., 2004) y los pesticidas (Barth et al., 1995) pueden incrementar la actividad de enzimas del metabolismo de fenoles, y como consecuencia, aumentar la susceptibilidad al pardeamiento de los productos.

Como ya se ha mencionado anteriormente, el SA y el MeJA son moléculas implicadas en sistemas de transducción de señales, que inducen enzimas que a su vez catalizan reacciones biosintéticas para formar compuestos de defensa tales como compuestos fenólicos, entre otros (Ding et al., 2002). Así, el pretratamiento de células de perejil con SA aumentó la expresión de los genes que codifican la enzima PAL y la 4-cumarato:CoA ligasa (Thulke y Conrath, 1998). Pastírová et al. (2004) observaron que la aplicación de SA inducía la acumulación de cumarinas en plantas de manzanilla (*Matricaria chamomilla* L.). En uvas, Chen et al. (2006) demostraron que el SA podría activar la enzima PAL aumentando la acumulación de su mensajero, la síntesis de proteína "de novo", la actividad enzimática y, en consecuencia, la acumulación de fenilpropanoides tales como ácidos fenólicos.

Estudios de la expresión de un gen *liPAL* aislado del tetraploide *Isatis indigotica* Fort., revelaron que las giberelinas, el ABA, el MeJA y los tratamientos con bajas

temperaturas, aumentaban los niveles de transcripción del mensajero (Lu et al., 2006). Además, se ha demostrado en zanahorias (Chalutz, 1973), cítricos (Riov et al., 1969, Lafuente et al., 2001) y manzanas (Faragher y Chalmers, 1977; Blankenship y Unrath, 1988), que el etileno es un regulador de la actividad PAL. La exposición de tejidos de plantas a niveles hormonales de etileno resultó en la inducción de la actividad PAL (Hyodo et al., 1991; Ritenour y Saltveit, 1996; Takahashi et al., 1996); y se ha observado que el daño mecánico puede también causar un aumento en la producción de etileno, con un correspondiente aumento en la actividad PAL (Ke y Saltveit, 1989). Asimismo, Martínez-Téllez y Lafuente (1997) sugirieron que el etileno podría estar implicado en el control de la actividad de la PAL en los frutos de mandarina Fortune almacenados a bajas temperaturas. Tratamientos realizados con 1-MCP, inhibidor de la acción del etileno, afectaron negativamente a la concentración de fenoles totales en la fruta madura. En este sentido, fresas tratadas con 1-MCP presentaron una disminución en la actividad PAL y la concomitante inhibición del incremento en la concentración de flavonoides y compuestos fenólicos (Jiang et al., 2001).

También ha sido muy estudiado el aumento de compuestos fenólicos como respuesta a la infección con distintos patógenos. Así, Arfaoui et al. (2007) comprobaron que el aumento en la acumulación de compuestos fenólicos, observado en la germinación de garbanzos inoculados con hongo del género *Fusarium*, podría ser atribuido a la mayor expresión de genes de la ruta de los fenilpropanoides, ocasionado por el pretratamiento con *Rhizobium*. En uva, se han detectado aumentos en el contenido de la fitoalexina resveratrol en hojas de vid y bayas infectadas por *B. cinerea* (Langcake y Mc Carthy, 1979; Jeandet et al., 1995). También se han visto aumentos en la expresión de los genes que codifican las enzimas PAL y STS en hojas de vid infectadas por *B. cinerea*, como mecanismo de

defensa de la planta frente al ataque por patógenos, pero la activación de estos genes no fue suficiente para detener la extensión del hongo (Bézier et al., 2002).

Por otro lado, los fenilpropanoides tienen un papel regulador sobre distintos procesos. Las moléculas mejor estudiadas en este sentido son los derivados glicosilados del dihidrokaempferol y de los flavonoides como potenciales reguladores de la división celular (Teutonico et al., 1991); los flavonoides como reguladores del transporte de auxinas (Jacobs y Rubery, 1988); y el SA como regulador de la expresión de genes que se inducen por infección de patógenos o por estrés oxidativo (Dempsey et al., 1999).

Dado que el metabolismo de fenilpropanoides es uno de los mecanismos de respuesta de las plantas frente a distintos estreses bióticos y abióticos, se estudió la implicación de enzimas claves de esta ruta (PAL, CHS y STS), así como sus productos derivados, antocianos y resveratrol, en la mejora de la calidad de la uva de mesa conservada a bajas temperaturas y altas concentraciones de CO_2.

ARTÍCULO 1

Effect of high CO_2 pretreatment on quality, fungal decay and molecular regulation of stilbene phytoalexin biosynthesis in stored table grapes

M[a] Teresa Sanchez-Ballesta, Jorge Bernardo Jiménez, Irene Romero, José Ma Orea , Roberto Maldonado, Ángel González Ureña, Ma Isabel Escribano, Carmen Merodio

Postharvest Biology and Technology 42 (2006) 209–216.

RESUMEN

Uvas de mesa (*Vitis vinifera*) cv. Cardinal almacenadas a baja temperatura fueron analizadas para determinar el efecto del pretratamiento con 20 kPa O_2 +20 kPa CO_2 +60 kPa N_2 durante 3 días sobre la calidad y el ataque por hongo. El patrón de la expresión génica de la estilbeno sintasa (STS) y los niveles de *trans*-resveratrol fueron también analizados en uvas durante el almacenamiento a bajas temperaturas (0ºC) y posteriormente durante el tiempo de vida útil a 20ºC durante 2 días. Nuestros resultados mostraron que el pretratamiento con alto CO_2 fue efectivo para mejorar la apariencia de los racimos y mantener la calidad de los frutos. En los racimos tratados con CO_2, los índices de marchitamiento y oscurecimiento, la disminución en el contenido relativo de agua y la pérdida de peso fueron también menores que en uvas no tratadas. Los niveles de mRNA de *STS* y la acumulación de *trans*-resveratrol en uvas tratadas con CO_2 fueron mucho más bajos que en uvas no tratadas durante el almacenamiento a bajas temperaturas. Además, el patrón de expresión génica de *STS* y el contenido de *trans*-resveratrol en uvas de mesa tratadas con CO_2 fueron consistentes con la reducción del ataque por hongo, crecido de forma natural, producida por este pretratamiento. Este tratamiento efectivo no estresante evita la inducción del *trans*-resveratrol durante el almacenamiento a bajas temperaturas hasta que su síntesis es aumentada durante el tiempo de vida útil a 20ºC.

Available online at www.sciencedirect.com
ScienceDirect
Postharvest Biology and Technology 42 (2006) 209–216
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

Effect of high CO_2 pretreatment on quality, fungal decay and molecular regulation of stilbene phytoalexin biosynthesis in stored table grapes

Mª Teresa Sanchez-Ballesta [a], Jorge Bernardo Jiménez [b], Irene Romero [a], José Mª Orea [b], Roberto Maldonado [a], Ángel González Ureña [b], Mª Isabel Escribano [a], Carmen Merodio [a,*]

[a] *Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frio, IF-CSIC, Ciudad Universitaria, E-28040 Madrid, Spain*
[b] *Unidad de Láseres y Haces Moleculares, Instituto Pluridiciplinar, Pº Juan XXIII, 1, E-28040 Madrid, Spain*

Received 17 March 2006; accepted 3 July 2006

Abstract

Table grapes (*Vitis vinifera*) cv. Cardinal stored at low temperature were analysed to determine the effect of pretreatment with 20 kPa O_2 + 20 kPa CO_2 + 60 kPa N_2 for 3 days on quality and control of decay. The pattern of stilbene synthase (STS) gene expression and trans-resveratrol levels were also analyzed in grapes during low temperature storage at 0 °C and further shelf-life at 20 °C for 2 days. Our results showed that high CO_2 pretreatment was effective for improving appearance of the bunches and maintaining the quality of the berries. In CO_2-treated bunches the browning and withering index, the decline in relative water content and the weight loss were also lower than in non-treated ones. The levels of *STS* mRNA and the accumulation of trans-resveratrol in CO_2-treated grapes were much lower than in the non-treated grapes during low temperature storage. Moreover, the pattern of *STS* gene expression and trans-resveratrol content in CO_2-treated grapes was consistent with the reduction of natural total decay produced by this pretreatment. This effective non-stressing treatment avoids the induction of trans-resveratrol during low temperature storage until its synthesis is enhanced during shelf-life at 20 °C.

Keywords: Table grapes; *Vitis vinifera*; Fruit quality; Postharvest technology; Carbon dioxide; Laser spectrometry; Stilbene synthase; Resveratrol

1. Introduction

Low temperature storage is one of the most effective technologies for extending the postharvest life of fruit and vegetables. However, in table grapes low temperature storage life is limited by high sensitivity to fungal attack, mainly from *Botrytis cinerea*. This pathogen, which has a considerable economic impact in horticulture, is usually controlled by means of fungicides, but this can lead to multiple resistance in the pathogen population (Raposo et al., 1996). Moreover, because postharvest chemical treatments are restricted in most countries, safe alternative control technologies need to be developed to assure high quality fruit and control fungal attack. Commercial alternatives to the use of SO_2 generators have been proposed to maintain the quality of table grapes over the short term, using modified atmosphere packaging (MAP) alone or in combination with natural fungicides (Artés-Hernández et al., 2006). Also, the application of controlled atmospheres (CA) under a continuous flow has been reported to be beneficial for controlling postharvest diseases in table grapes for prolonged cold storage (Yahia et al., 1983). However, rachis browning places a limitation on prolonged storage under CA (Crisosto et al., 2002; Retamales et al., 2003). An alternative gaseous treatment would be pretreatment with high CO_2 concentrations for shorter storage periods during postharvest handling of table grapes. Considering the low risk of this treatment, the use of pretreaments with high CO_2 levels to control *Botrytis* storage rot in maintaining table grape quality could be an interesting area of research. However, we need to know the efficacy of pretreatment with high CO_2 levels in controlling decay and maintaining quality of both rachis and berries. We also need to know whether this treatment corre-

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: merodio@if.csic.es (C. Merodio).

0925-5214/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.postharvbio.2006.07.002

lates with the activation of a number of defense mechanisms in grapes. In previous work, we reported that the expression of *Vitis* class I chitinase and β-1,3-glucanase genes is not enhanced with CO_2 treatments which control fungal decay, and we suggested that the efficacy of high CO_2 pretreatment on the reduction of fungal decay is not mediated by the induction of the above mentioned PR genes (Romero et al., 2006).

Among the other possible defense mechanisms, the induction of the phenylpropanoid pathway appears to play a crucial role (Hahlbrock and Scheel, 1989). In grapes, the production of phytoalexins such as stilbenes on the phenylalanine/polymalonate pathway (Langcake and Pryce, 1977) is one of the most important defense pathways. Phytoalexins from *Vitis* species are composed of a restricted group of molecules belonging to the stilbene family and deriving primarily from trans-resveratrol (3,4′-5-trihydroxystilbene). Trans-resveratrol, which has recently received a lot of attention because of its implication in human health (Bertelli et al., 1995; Jang et al., 1997; Hung et al., 2000), is mainly present in the skin of berries. Resveratrol production depends on many factors and the concentration is highly dependent on the cultivar. In grape tissues, trans-resveratrol production has been described after microbial infection or treatment with elicitors and several kinds of stress (Langcake and Pryce, 1976; Liswidowati et al., 1991; Jeandet et al., 1995). Specifically, production of trans-resveratrol in wine and table grapes in response to UV-C irradiation has been extensively studied (Creasy and Coffee, 1988; Langcake and Pryce, 1977; Cantos et al., 2002). Moreover, a good correlation has been reported between resveratrol production (as induced by UV-C elicitation) and grey mould resistance (Sbaghi et al., 1995), and also black mould caused by *Rhizopus stolonifer* on several table grape varieties (Sarig et al., 1997). Trans-resveratrol is synthesized by condensation of one molecule of 4-coumaroyl-CoA with three malonyl-CoA units in a reaction catalyzed by stilbene synthase (STS). This enzyme is encoded by a multigene family characterized by high nucleotide sequence homology (Melchior and Kindl, 1990; Wiese et al., 1994). A constitutive expression of *STS* has been reported (Sparvoli et al., 1994), but it could be expressed differentially in response to biotic and abiotic stresses (Preisig-Müller et al., 1999; Brehm et al., 1999; Versari et al., 2001).

The aim of this work was to analyze the effectiveness of pretreatment with 20% CO_2 in controlling natural postharvest decay and its effect on quality attributes of rachis and berries and the molecular regulation of stilbene phytoalexin biosynthesis. *STS* gene expression and trans-resveratrol content of table grapes cv. Cardinal during low temperature storage at 0 °C and further shelf-life at 20 °C for 2 days were monitored. These technological and molecular studies were based on the premise that an understanding of the role of high CO_2 levels in disease resistance and quality may contribute to the development of more effective postharvest technologies.

2. Materials and methods

2.1. Plant material

Table grapes (*Vitis vinifera* L. cv. Cardinal) were harvested at random in Camas (Sevilla, Spain) in July. Early-harvesting mature berries were used in this work (12.7% total soluble solids; 0.81% tartaric acid). After harvesting, field-packaged bunches were transported to the laboratory, where fruit were immediately forced-air precooled for 14 h at −1 °C. After cooling, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 °C and 95% relative humidity (RH) in two sealed neoprene containers of 1 m^3 capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container. One lot was stored under normal atmosphere for 33 days (non-treated fruit) and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. The CO_2 concentration was maintained throughout the pretreatment experiment and was measured daily using an automated gas chromatograph system equipped with a thermal conductivity detector and Poraplot Q column (Varian Chrompack CP20033P). After 3 days, CO_2-treated grapes were transferred to air under the same conditions as the non-treated fruit until the end of the storage period. At the end of low temperature storage (33 days), both CO_2-treated and non-treated grapes were transferred to ventilated storage containers for an additional 2 days at 20 °C and 95% RH, to simulate shelf-life during marketing. Ten clusters were sampled periodically during low temperature storage and at the end of their shelf-life. Berries obtained from five clusters (approximately 300 g each cluster) were peeled and the skin was frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis. For quality parameters, 45 berries were used, randomly removed from five clusters and distributed in three replicates of 15 berries each.

2.2. Quality assessments and total decay

Bunch withering, browning indexes and the relative water status (RWC) of the stem were determined for each bunch. Bunch withering was determined using the following subjective scale: (0) none, (1) onset of withering in pedicel and apex of stem, (2) withering of pedicel, apex of stem and over 10% of the main stem, (3) withering in up to 50% of main stem, and (4) total withering of main stem. The scale used for the browning index was: (0) none, (1) slight, (2) moderate, (3) severe, and (4) extreme. The water status of the stem was followed by measuring the RWC. One centimeter of stem, cut with a razor blade was weighed fresh, again after 24 h rehydration with distilled water at room temperature, and finally after drying at 85 °C, to give the fresh (FW), turgid (TW) and dry (DW) weights, respectively. RWC was determined from the equation: $RWC\ (\%) = (FW - DW)/(TW - DW) \times 100$.

Berry quality assessment considered dry matter (DM), soluble solids content (SSC), pH, titratable acidity (TA) maturity

index (ratio SSC/TA), colour and total decay. The DM content (%) was determined in grapes cut longitudinally with a razor blade in four pieces, after drying at 65 °C to a stable weight after two subsequent weight measurements. SSC was determined using a digital refractometer Atago PR-101 (Atago Co. Ltd., Japan) at 20 °C and expressed in °Brix. The pH of the juice was measured in a pH meter with glass electrode. TA was determined by titration with 0.1N NaOH up to pH 8.1 and results were expressed in % tartaric acid.

The external colour of the berries was measured using the Hunter Lab System and a Minolta colorimeter CR200^{TM} model (Minolta Camera Co., Osaka, Japan). The color space coordinates L^*, a^*, b^* and chroma (C^*) $(a^{*2} + b^{*2})^{1/2}$ were determined around the equatorial region in three different positions.

Total anthocyanins were extracted from frozen skin samples (0.5 g) four times with 0.5 mL methanol acidified with 0.01% (v/v) HCl under continuous ultra-sonication for 10 min and centrifuged at 2000 × *g* for 10 min each time. All steps were carried out at 4 °C. Anthocyanins contents were measured in the combined supernatant by the pH differential method (Wrolstad, 1976). The absorbance was measured with a Perkin-Elmer spectrophotometer (Norwalk, CT) at 510 and 700 nm in buffers at pH 1.0 and 4.5, using $A = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}$ with a molar extinction coefficient of malvidin-3-glucoside of 28,000 L mol^{-1} cm^{-1}. The results were expressed as mg of malvidin-3-glucoside equivalent per g fresh weight.

Total storage decay was assessed on the basis of the total decay after removing and weighing the healthy berries. The weight of the decayed berries was calculated by subtracting healthy berries from the total cluster weight. Thus, total decay was expressed as the percentage of decayed berries with respect to the original cluster weight.

2.3. Northern blot hybridization

Total RNA was extracted from the skin of grapes according to the method of Salzman et al. (1999). Samples of denatured total RNA (10 μg) were separated on 1.2% (w/v) agarose-formaldehyde gels. RNAs were transferred to Hybond-N membrane (USB, Amersham) using 20 × SSC and cross-linked using a UV Stratalinker 800 (Stratagene La Jolla, CA). Equal loading was confirmed by ethidium bromide staining and by membrane staining with methylene blue.

A partial cDNA clone of stilbene synthase was obtained by RT-PCR. cDNA synthesis was performed with 5 μg of total RNA from the skin of grapes treated with CO_2 and stored for 12 days at 0 °C. The reaction was carried out in the presence of 500 ng of oligo-dT with 100 units of reverse transcriptase (Ecogen). A stilbene synthase gene DNA fragment was obtained by PCR amplification using the cDNA as template and the combination of the sense primer 5′-CCAAACATTGGTGCTTATATGGCTC-3′ and the antisense primer 5′-AGGGATTTCTTTCTCATCTCATCCA-3. The PCR product was cloned into the pGEMT vector (Promega) and confirmed by sequencing.

The partial DNA probe was random-primer labelled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 °C in 7% sodium dodecyl sulphate, 0.33 M phosphate buffer, pH 7.2, and 1 mM EDTA, then washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 0.1 × SSC, 0.1% SDS at 65 °C and exposed to Kodak X-Omat SX film at −80 °C. Autoradiographs were digitally scanned and band densities quantified by image densitometry using Scion Image software (Scion Corporation, Frederick, MD). The 100% was assigned to the maximum optical density value achieved in each Northern and the rest of the optical densities were normalized to the maximum value and expressed as percentage of relative accumulation (RA).

2.4. Laser analytical technique

Skin samples (0.5 g) homogenized in liquid nitrogen were extracted with 0.5 mL methanol at room temperature for 10 min with ultra-sonication for four times. The extracts were centrifuged, filtered and protected from light to avoid *cis*-isomerization of stilbenes. The experimental analysis of resveratrol from grape skin has already been described elsewhere (Montero et al., 2000, 2003; Orea et al., 2001), so only a brief description is given here. Essentially, it is based on the combination of laser desorption (LD) with resonance-enhanced multi-photon ionization (REMPI) coupled with time-of-flight mass spectrometry (TOF-MS) detection. It consists of two independent high vacuum chambers; the first chamber was used for both laser desorption and laser post-ionization of the sample followed by acceleration of the ions towards the second chamber, basically a time-of-flight unit with a twin-microchannel plate detector. The sample was deposited on a Pyrex disc, which was parallel to the first accelerating plate. A step motor was used to control disc rotation. The whole sample holder and motor assembly was attached to a linear translator to allow adjustment of the perpendicular direction to the desorption laser. This movement changes the rotation radius so as to increase the number of runs for a given sample.

First harmonic pulses of 5 ns duration from a Nd:YAG laser (1064 nm) were used for sample desorption, and a frequency-doubled dye laser was used to ionize the desorbed neutrals. A system for frequency-doubling the output of the dye laser and active wavelength laser scanning (INRAD-AT-III-UV) with a doubling crystal (BBO-TST) was used to scan the output from 235 up to 365 nm in search of the resonant wavelength of the target analyte. The wavelength used for analysis of trans-resveratrol was 302.1 nm, determined as the optimal wavelength for trans-resveratrol analysis in complex samples. The ions produced by the second laser pulse were accelerated towards the TOF tube by a Whiley–McLaren type acceleration system and detected by a twin-microchannel plate detector (Comstock CP-625C/50F) placed at the end of the TOF tube.

2.5. Statistical analyses

Data from at least three replicates per sample were subjected to analysis of variance (ANOVA) (Statgraphics program, STSC, Rockville, MD). Multiple variance analysis was employed to determine the significance of the data at $P \leq 0.05$.

3. Results

3.1. Changes in quality of bunches and in total decay in CO_2-treated and non-treated table grapes stored at 0 °C

As seen in Table 1 non-treated bunches presented a greater increase in the withering index at the end of low temperature storage than CO_2-treated bunches. With respect to the browning index of the rachis, an increase was observed in non-treated bunches while in CO_2-treated bunches the browning index was lower, indicating that CO_2 treatment was effective in maintaining the visual appearance of the rachis. Also, in non-treated bunches a significant decline in RWC was recorded under storage at low temperature in air. The decline in RWC was less in CO_2-treated fruit after 33 days. The beneficial effects of CO_2 treatment on increasing better appearance could be explained by the lower water loss of the rachis under this treatment. Moreover, CO_2-treated bunches lost less weight than did non-treated bunches. With respect to berry quality, we observed that at the end of low temperature storage, DM of CO_2-treated grapes did not change with respect to freshly harvested fruit, while a slight increase was recorded in non-treated grapes. SSC and TA at harvest were 12.85 and 0.843, respectively and the SSC/TA ratio was 15.24. This ratio increased in both non-treated and CO_2-treated fruit during cold storage, reaching values of 20.23 and 18.43, respectively. No major differences were observed for SSC between non-treated and CO_2-treated grapes after 33 days of storage. As regards the external colour of the berries, no browning occurred in CO_2-treated fruit. CO_2 treatment maintained the bright skin colour of harvested grapes better, with higher C^* value than non-treated grapes. The total content of the anthocyanins found at harvest was 1.39 mg g FW^{-1} (Table 1). Both non-treated and CO_2-treated grapes exhibited an increase in total anthocyanins, but this increase was significantly higher in non-treated grapes. In CO_2-treated fruit the total decay incidence was significantly lower than in non-treated grapes (Table 1). After 33 days of storage at 0 °C, 25.5% of total decay was quantified in non-treated grapes, while 5.0% diseased berries were found in CO_2-treated clusters.

Table 1
Withering index, browning index, relative water content (RWC), weight loss, dry matter (DM), soluble solids content (SSC), titratable acidity (TA), maturity index, pH, color parameters (L^*, a^* and C^*), total anthocyanins and total decay of grapes cv. Cardinal treated with 20% CO_2 and stored during 33 days at 0 °C

Quality parameters	After-harvest	33 days of storage at 0 °C	
		Non-treated	CO_2-treated
Withering index[a]	0	3.33	1.50
Browning index[b]	0	2.83	1.67
Relative water content (%)	88.78 a	56.52 b	76.97 ab
Weight loss (%)	0	7.42 a	3.93 b
Dry matter (%)	14.71 a	15.41 a	14.96 a
SSC (%)	12.85 a	14.10 b	14.28 b
TA (% tartaric acid)	0.84 a	0.69 b	0.77 c
Maturity index (SSC/TA)	15.24	20.23	18.43
pH	3.33 a	3.69 b	3.64 b
L^*	35.95 a	24.56 b	23.22 b
C^*	16.89 a	2.84 b	4.07 c
Total anthocyanins (mg g FW^{-1})	1.39 a	1.85 b	1.57 a
Total decay	0	25.50 a	5.00 b

[a] Scale of withering: 0, none; 1, onset of withering in pedicel and apex of bunch; 2, withering of pedicel, apex of bunch and over 10% of the main stem; 3, withering in up to 50% of main stem; 4, total withering of main stem.
[b] Scale of browning: 0, none; 1, slight; 2, moderate; 3, severe; 4, extreme
Means within files with different letters denotes statistically significant differences (LSD = 95%).

3.2. Effect of high CO_2 treatment on trans-resveratrol content during low temperature storage of grapes

Fig. 1 shows the change in the trans-resveratrol content in treated and non-treated samples obtained by LD + REMPI-TOFMS. In freshly harvested table grapes the trans-resveratrol content was 44.11 μg g FW^{-1}. While trans-resveratrol levels in CO_2-treated fruit showed a steady increase after 22 days at 0 °C, the non-treated grapes presented a sharp accumulation after 33 days of storage, reaching values of 69.89 μg g FW^{-1}. At this time, the trans-resveratrol content in the CO_2-treated grapes was only ca. 60% that of the non-treated grapes, with a value of 42.11 μg g FW^{-1}.

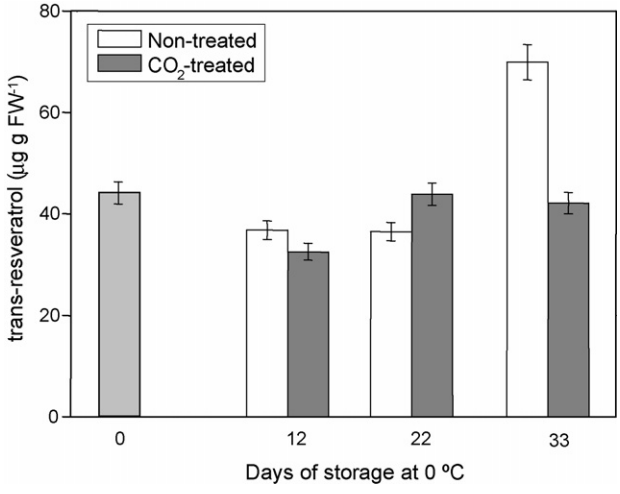


Fig. 1. Changes in trans-resveratrol content in the skin of CO_2-treated and non-treated 'cardinal' table grapes during 33 days of storage at 0 °C (see text for details). Values are the means of three replicate samples ± S.E.

3.3. Effect of high CO_2 treatment on the accumulation of STS mRNA during low temperature storage of grapes

To determine whether low temperature storage may affect the pattern of *STS* gene expression in 'cardinal' grapes, and how low-temperature treatment with high CO_2 concentration affected transcript accumulation, the total mRNA prepared from the skin of CO_2-treated and non-treated grapes stored at 0 °C for up to 33 days was analyzed by Northern hybridization (Fig. 2). A partial cDNA clone of 773-bp of the stilbene synthase obtained by RT-PCR (GenBank Accession Number DQ235274) was used as a probe. The storage of grapes at 0 °C induced *STS* mRNA accumulation in both non-treated and CO_2-treated grapes. However, *STS* mRNA levels in the non-treated grapes were higher than in CO_2-treated fruit. In non-treated grapes there was a slow but progressive accumulation of *STS* transcript, which reached a maximum by day 28 and decreased slightly thereafter. However, the *STS* transcript in CO_2-treated grapes remained at stable low levels until the end of storage.

3.4. STS mRNA accumulation and trans-resveratrol content during the shelf-life of CO_2-treated and non-treated grapes at 20 °C

To simulate shelf-life during marketing of grapes, CO_2-treated and non-treated fruit stored for 33 days at 0 °C were transferred to 20 °C for 2 days and changes in the *STS* gene expression and trans-resveratrol content were analyzed. After 2 days at 20 °C, there was a sharp increase in the *STS* mRNA level in both treated and non-treated grapes, which was 35% greater in the non-treated fruit (Fig. 3). It is interesting to note that *STS* mRNA levels in CO_2-treated grapes did not increase during storage at 0 °C as occurred with non-treated grapes, where the accumulation of transcript was greater except for a decrease observed after 33 days of storage (Fig. 2). Upon transfer, the trans-resveratrol content was also higher in the skin tissues of non-treated grapes than in the treated ones. However, as noted earlier, at the beginning of this shelf-life

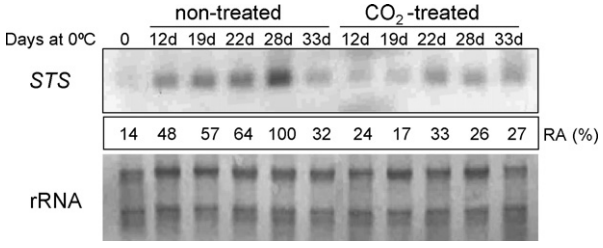


Fig. 2. Accumulation of *STS* mRNA in the skin of CO_2-treated and non-treated Cardinal table grapes stored for up to 33 days at 0 °C. 10 μg of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *STS* probe. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical densities values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining.

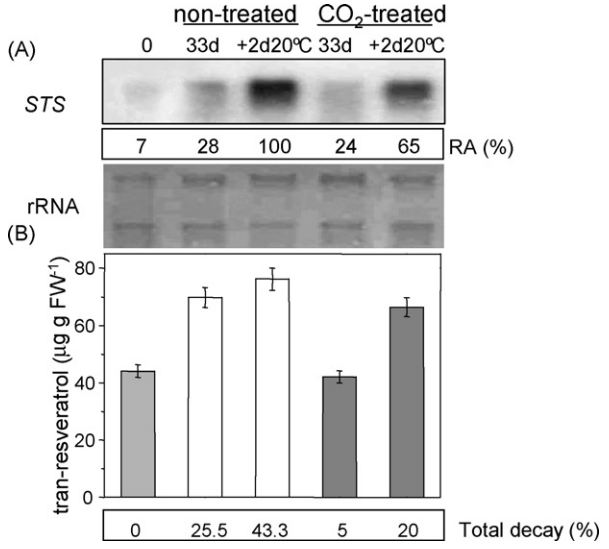


Fig. 3. Accumulation of *STS* mRNA (A) and trans-resveratrol (B) in the skin of CO_2-treated and non-treated Cardinal table grapes stored for 33 days at 0 °C and transferred to 20 °C for 2 days. (A) 10 μg of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *STS* probe. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical densities values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. (B) Trans-resveratrol values are the means of three replicate samples ± S.E.

period (after 33 days of storage), the trans-resveratrol content in the CO_2-treated grapes was only 55% of that observed in non-treated grapes. Thus, the trans-resveratrol increase rate during the shelf-life period was much more pronounced in the CO_2-treated samples: i.e., 72% versus only 10% in the non-treated samples. When table grapes were transferred to 20 °C for 2 days, although the levels of total decay increased sharply in both non-treated and CO_2-treated grapes, the increase rate was also much more pronounced in the CO_2-treated samples.

4. Discussion

During postharvest storage of table grapes serious problems arise from stem withering and browning which are detrimental to the visual appearance of the clusters and even determine table grape storability. A considerable deterioration in the visual appearance of non-treated clusters was observed after 33 days of storage at 0 °C (Table 1). The withering and browning indexes at the end of the storage were consistently lower in bunches pretreated with 20% CO_2 for 3 days than in non-treated bunches, with values of 1.50 and 3.33, respectively. On the other hand, high stem browning development has been reported after 2 months under CA treatments using CO_2 levels ≥15 kPa (Retamales et al., 2003). The beneficial effects of pretreatment of CO_2 on appearance of bunches could be explained by lower water loss from the rachis under this treatment. In the present work we analyzed the RWC of

the stem as an alternative measure of water status. The RWC is a measure of the actual water content relative to the water content at full turgidity, reflecting the metabolic activity in tissues (Flower and Lidlow, 1986). We observed that stem RWC in non-treated bunches declined sharply as a result of storage at 0 °C. In CO_2-treated stems, on the other hand, the lower increase in internal water content during rehydration could indicate an osmotic adjustment, which prevents perturbation of cell metabolism caused by low temperature. Also, many plant species during water stress conditions shows a decline in RWC (Nagy et al., 1995), including fruit tissues (Walter et al., 1990). The results showed that weight loss in CO_2-treated grapes was lower than in non-treated grapes after 33 days of storage (3.93 and 7.42, respectively, Table 1).

As regards berry quality, it is widely accepted that the most important parameters determining consumer acceptability of table grapes are bright red colour and the ratio between SSC and TA. No significant changes were observed for SSC between non-treated and CO_2-treated grapes after 33 days of storage, although SSC did increase significantly with respect to freshly harvested fruit. Since berries were collected at 12.85%, in an early harvest state, such an increase of soluble solids could be the result of normal postharvest metabolic activity. This accumulation was also reflected in the change in the SSC/TA ratio, which was lower in CO_2-treated fruit, indicating that this treatment delay the metabolic changes associated with maturation, so that development was less pronounced in CO_2-treated grapes than non-treated grapes. These results are consistent with previous work, which suggested that high CO_2 levels may act via physiological response of the fruit tissue to delay ripening and senescence of green tissues (Del Cura et al., 1996). It has been reported that in general, SSC, TA and pH values remained quite constant in several varieties of grapes stored at 0 °C under several Ca conditions (Crisosto et al., 2002; Artés-Hernández et al., 2006). Also, CO_2 treatment maintained berry skin colour better during cold storage, and no colour anomalies were found in CO_2-treated fruit; this is consistent with the findings of Cimino et al. (1987) in the white 'Italia' cultivar stored for 99 days at 0 °C under CA. With respect to the effect of pretreatment with high CO_2 levels on polyphenol compounds, we observed (Table 1) that at the end of the storage period the total anthocyanins content in the skin tissues was higher in non-treated than in CO_2-treated grapes.

A sharp rise in trans-resveratrol levels measured by REMPI-TOFMS took place in non-treated grapes after 33 days of storage at 0 °C. However no increase in trans-resveratrol content was observed in the skin tissues of CO_2-treated grapes. It has been reported that the content of trans-resveratrol in the skin of berries increased significantly in response to several stresses, such as O_3 shock, UV-C irradiation and pathogen infection (Creasy and Coffee, 1988; Sarig et al., 1997; Cantos et al., 2002). Our results show that at the end of the storage period, the highest trans-resveratrol content was detected in non-treated grapes, reaching values of 69.89 μg g FW^{-1}. In CO_2-treated grapes the trans-resveratrol content was a ca. 60% that of non-treated grapes reaching values of 42.11 μg g FW^{-1} (Fig. 1).

The differences observed between the CO_2-treated and non-treated grapes in trans-resveratrol levels were also observable in the levels of *STS* transcript (Fig. 2), demonstrating that inducible stilbene accumulation in skin berries is highly regulated at the level of *STS* gene expression. We have isolated a partial *STS* cDNA clone, and nucleotide and amino acid sequence analyses revealed that the encoded STS share significant levels of identity with STS sequences from *Vitis* (Melchior and Kindl, 1990; Wiese et al., 1994). *STS* mRNA levels in the non-treated grapes, which reached a maximum by day 28, were nonetheless substantially higher than in CO_2-treated fruit. It has been extensively studied that the expression of *STS* genes is induced in response to biotic and abiotic stresses such as pathogen infection (Preisig-Müller et al., 1999), ozone (Zinser et al., 2000) and UV-light (Versari et al., 2001). Pretreatment with high CO_2 levels appears to be a clear effect avoiding the induction of *STS* genes in CO_2-treated grapes during low temperature storage. In these treated grapes *STS* mRNA levels slightly increased and remained at stable state low levels until the end of storage. In a similar way, trans-resveratrol content in these CO_2-treated tissues remained virtually unchanged with respect to those quantified in freshly harvested grapes. Moreover, as we indicated above, no significant increase in total fungal decay was observed in CO_2-treated grapes (Table 1). After shelf-life at 20 °C, while trans-resveratrol content increased to 72% in CO_2-treated fruit, it only reached 10% in non-treated fruit. This high potentiality for synthesizing resveratrol in CO_2-treated grapes during shelf-life was similar to that of non-treated fruit at the end of storage at 0 °C. An increase has also been reported in resveratrol content (1.2 ± 0.6 μg g FW^{-1} of grape samples at harvest) during shelf-life of table grapes stored under CA (Artés-Hernández et al., 2003). Our results suggest that *STS* gene expression and resveratrol accumulation are not associated with the reduction of fungal decay. Moreover, a further possible factor involved in this response is the early state of maturity of this red table grape variety. In this connection, a clear negative correlation has been reported between the resveratrol content of grape skin and the developmental stage of berries measured by the sugar concentration of the juice (Jeandet et al., 1995).

We conclude that the range of negative effects occurring in non-treated fruit stored at 0 °C, mostly increased withering and browning indices, water loss and total decay, were reduced by pretreatment with high CO_2 levels, thus improving the appearance of table grapes. Likewise, the CO_2 treatment consistently restrained the susceptibility of berries to total decay and the accumulation of *STS* mRNA and trans-resveratrol content. Our results indicate that the efficacy of high CO_2 pretreatment in reducing total fungal decay during low temperature storage is not mediated by induction of *STS* gene expression. Furthermore, these results point to new aspects of research aimed to identify responses in table grapes associated with low temperature storage and mechanism,

that confer a competitive advantage on CO_2-treated table grapes.

Acknowledgements

This work was supported by research grants (AGL2005-04502/ALI and AGL2002-02308) from the CICYT (Spain). MT.S.-B. and R.M. were supported by a postdoctoral contract from the Madrid Region and by a fellowship from the MCyT (Spain), respectively. The Instituto Pluridisciplinar group wishes to thank the MCyT for financial support (grant CTQ2004-03468/BQUI). The authors are grateful to Frutaria SAT for providing table grapes and to Agrotecnica. We also wish to thank Prof. Jose Luis De La Plaza and Dr. Joston Simao de Assis for scientific and technological advices.

References

Artés-Hernández, F., Artés, F., Tomás-Barberán, F.A., 2003. Quality and enhancement of bioactive phenolics in Cv. Napoleon table grapes exposed to different postharvest gaseous treatments. J. Agric. Food Chem. 51, 5290–5295.

Artés-Hernández, F., Tomás-Barberán, F.A., Artés, F., 2006. Modified atmosphere packaging preserves quality of SO_2-free 'Superior seedless' table grapes. Postharvest Biol. Technol. 39, 146–154.

Bertelli, A.A., Giovannini, L., Giannessi, D., Migliori, M., Bernini, W., Fregoni, M., Bertelli, A., 1995. Antiplatelet activity of synthetic and natural resveratrol in red wine. Int. J. Tissue React. 17, 1–3.

Brehm, I., Preisig-Müller, R., Kindl, H., 1999. Grapevine protoplasts as a transient expression system for comparison of stilbene synthase genes containing cGMP-responsive promoter elements. Z. Naturforsch. (C) 54, 220–229.

Cantos, E., Espin, J.C., Tomás-Barberán, F.A., 2002. Postharvest stilbene-enrichment of red and white table grape varities using UV-C irradiation pulses. J. Agric. Food Chem. 50, 6322–6329.

Cimino, A., Mari, M., Marchi, A., 1987. ULO storage of table grapes and kiwifruit.ULO storage of table grapes and kiwifruit. In: Proceedings of the XVIIth International Congress on Refrigeration, Vienna, pp. 642–646.

Creasy, L.L., Coffee, M., 1988. Phytoalexin production potential of grape berries. J. Am. Soc. Hort. Sci. 113, 230–234.

Crisosto, C.H., Garner, D., Crisosto, G., 2002. Carbon dioxide-enriched atmospheres during cold storage limit losses from *Botrytis* but accelerate rachis browning of 'Redglobe' table grapes. Postharvest Biol. Technol. 26, 181–189.

Del Cura, B., Escribano, M.I., Zamorano, J.P., Merodio, C., 1996. High carbon dioxide delays postharvest changes in RnBPCase and polygalacturonase-related protein in cherimoya peel. J. Am. Soc. Hort. Sci. 121, 735–739.

Flower, D.J., Lidlow, M.M., 1986. Contribution of osmotic adjustment to the dehydration tolerance of water stressed pigeon pea (*Cajanas cajan (L)* Millsp) leaves. Plant Cell Environ. 9, 33–40.

Hahlbrock, K., Scheel, D., 1989. Physiology and molecular biology of phenylpropanoid metabolism. Ann. Rev. Plant Physiol. Plan Mol. Biol. 40, 347–369.

Hung, L.M., Chen, J.K., Huang, S.S., Lee, R.S., Su, M.J., 2000. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovasc. Res. 47, 549–555.

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C., Pezzuto, J.M., 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275, 218–220.

Jeandet, P., Bessis, R., Sbaghi, M., Meunier, P., 1995. Production of the phytoalexin resveratrol by grape as a response to *Botrytis cinerea* attacks under natural conditions. J. Phytopathol. 143, 135–139.

Langcake, P., Pryce, R.J., 1976. The production of resveratrol by *Vitis vinifera* and other members of the *Vitaceae* as a response to infection or injury. Physiol. Plant Pathol. 9, 77–86.

Langcake, P., Pryce, R.J., 1977. A new class of phytoalexins from grapevines. Experientia 33, 151–152.

Liswidowati, M.F., Hohmann, F., Burkhardt, S., Kindl, H., 1991. Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. Planta 183, 307–314.

Melchior, F., Kindl, H., 1990. Grapevine stilbene synthase cDNA only slightly differing from chalcone synthase cDNA is expressed in *Escherichia coli* into a catalytically active enzyme. FEBS Lett. 268, 17–20.

Montero, C., Orea, J.M., Muñoz, M.S., Lobo, R.F., González-Ureña, A., 2000. Nonvolatile analysis in fruits by laser resonant ionization spectrometry: application to resveratol in grapes. Appl. Phys. B 71, 601–605.

Montero, C., Cristescu, S.M., Jiménez, J.B., Orea, J.M., te Lintel Hekkert, S., Harren, F.J., González-Ureña, A., 2003. trans-Resveratrol and grape disease resistance. A dynamical study by high-resolution laser-based techniques. Plant Physiol. 131, 129–138.

Nagy, Z., Tuba, Z., Zsoldos, F., Erdei, L., 1995. CO_2 exchange and water relation responses of Sorhum and maize during water and salt stress. J. Plant Physiol. 145, 539–544.

Orea, J.M., Montero, C., Jiménez, J.B., González-Ureña, A., 2001. Analyses of trans-resveratrol by laser desorption coupled with resonant ionization spectrometry. Application to trans-resveratrol content in vine leaves and grape skin. Anal. Chem. 73, 5921–5959.

Preisig-Müller, R., Schwekendiek, A., Brehm, I., Reif, H.J., Kindl, H., 1999. Characterization of a pine multigene family containing elicitor-responsive stilbene synthase genes. Plant Mol. Biol. 39, 221–229.

Raposo, R., Delcan, J., Gomez, V., Melgarejo, P., 1996. Distribution and fitness of isolates of *Botrytis cinerea* with multiple fungicide resistance in Spanish greenhouses. Plant Pathol. 45, 497–505.

Retamales, J., Defilippi, B.G., Arias, M., Castillo, P., Manríquez, D., 2003. High-CO_2 controlled atmospheres reduce decay incidence in Thompson seedless and red globe table grapes. Postharvest Biol. Technol. 29, 177–182.

Romero, I., Sanchez-Ballesta, M.T., Maldonado, R., Escribano, M.I., Merodio, C., 2006. Expression of class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO_2 pretreatment. Postharvest Biol. Technol. 41, 9–15.

Salzman, R.A., Fujita, T., Zhu-Salzman, K., Hasegawa, P.M., Bressan, R.A., 1999. An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol. Biol. Rep. 17, 11–17.

Sarig, P., Zutkhi, Y., Monjauze, A., Lisker, N., Ben-Arie, R., 1997. Phytoalexin elicitation in grape berries and their susceptibility to *Rhizopus stolonifer*. Physiol. Mol. Plant Pathol. 50, 337–347.

Sbaghi, M., Jeandet, P., Faivre, B., Bessis, R., Fournioux, J.C., 1995. Development of methods using phytoalexin (resveratrol) assessment as a selection criterion to screen grapevine *in vitro* cultures for resistance to grey mould (*Botrytis cinerea*). Euphytica 86, 41–47.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G., Tonelli, C., 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol. Biol. 24, 743–755.

Versari, A., Parpinello, G.P., Tornielli, G.B., Ferrarini, R., Giulivo, C., 2001. Stilbene compounds and stilbene synthase expression during ripening, wilting, and UV treatment in grape cv. Corvina. J. Agric. Food Chem. 49, 5531–5536.

Walter, W.M., Epley, D.G., McFeeters, R.F., 1990. Effect of water-stress on stored pickling cucumbers. J. Agric. Food Chem. 38, 2185–2191.

Wiese, W., Vornam, B., Krause, E., Kindl, H., 1994. Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine DNA fragment. Plant Mol. Biol. 26, 667–677.

Wrolstad, R.E., 1976. Color and pigment analysis in fruit products (Bulletin 624). Corvallis, Oregon: Oregon Agricultural Experimental Station.

Yahia, E.M., Nelson, K.E., Kader, A.A., 1983. Postharvest quality and storage life of grapes as influenced by adding carbon monoxide to air or controlled atmospheres. J. Am. Soc. Hort. Sci. 108, 1067–1071.

Zinser, C., Jungblut, T., Heller, W., Seidlitz, H.K., Schnitzler, J.P., Ernst, D., Sandermann, H., 2000. The effect of ozone in Scots pine (*Pinus sylvestris* L.): gene expression, biochemical changes and interactions with UV-B radiation. Plant Cell Environ. 23, 975–982.

ARTÍCULO 2

Involvement of the phenylpropanoid pathway in the response of table grapes to low temperature and high CO_2 levels.

M[a] Teresa Sanchez-Ballesta, Irene Romero, Jorge Bernardo Jiménez, José M[a] Orea, Ángel González-Ureña, M[a] Isabel Escribano, Carmen Merodio.

Postharvest Biology and Technology 46 (1): 29-35

RESUMEN

Hemos analizado las respuestas inducidas por el almacenamiento a bajas temperaturas (0ºC) en uva de mesa roja (*Vitis vinifera* cv. Cardinal) y también los efectos de un tratamiento con 3 días de altos niveles de CO_2 (20% CO_2 plus 20% O_2). A nivel transcripcional, hemos estudiado las enzimas clave de la ruta de los fenilpropanoides, L-fenilalanina amonio-liasa (PAL), chalcona sintasa (CHS) y estilbeno sintasa (STS), junto con los productos finales de algunas de estas enzimas, como el *trans*-resveratrol y antocianos totales, y su implicación en la capacidad antioxidante. Los resultados, usando cDNAs parciales previamente aislados, indicaron que el almacenamiento a 0ºC durante 3 días aumentó los niveles de mRNA de *VcPAL*, *VcSTS* y *VcCHS* en la piel de uvas no tratadas, disminuyendo ligeramente en adelante. Por el contrario, la acumulación de estos transcritos fue menor en la piel de uva después de 3 días de tratamiento con CO_2, y fueron indetectables cuando la fruta tratada fue transferida a aire. Las bajas temperaturas modularon los niveles de antocianos totales y la capacidad antioxidante de uvas no tratadas, y redujeron el contenido de *trans*-resveratrol en uvas tratadas y no tratadas, aunque la disminución fue mayor en uvas tratadas con CO_2.

El conjunto de estos resultados indicaron que las uvas tratadas con CO_2 podrían ser menos sensibles a cambios en la temperatura durante las primeras etapas de almacenamiento a 0ºC, reduciendo los niveles de expresión de genes de la ruta de los fenilpropanoides y la acumulación de antocianos totales y capacidad antioxidante.

Available online at www.sciencedirect.com
ScienceDirect
Postharvest Biology and Technology 46 (2007) 29–35
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

Involvement of the phenylpropanoid pathway in the response of table grapes to low temperature and high CO_2 levels

Mª Teresa Sanchez-Ballesta [a,*,1], Irene Romero [a,1], Jorge Bernardo Jiménez [b], José Mª Orea [b], Ángel González-Ureña [b], Mª Isabel Escribano [a], Carmen Merodio [a]

[a] *Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frio, IF-CSIC, Ciudad Universitaria, E-28040 Madrid, Spain*
[b] *Unidad de Láseres y Haces Moleculares, Instituto Pluridiciplinar, P° Juan XXIII, 1, E-28040 Madrid, Spain*

Received 18 December 2006; accepted 6 April 2007

Abstract

We have analyzed the responses induced by low temperature storage (0 °C) in red table grape (*Vitis vinifera* L. cv. Cardinal) and also the effect of a 3 day treatment of high CO_2 levels (20% CO_2 plus 20% O_2). At the transcriptional level we studied the key phenylpropanoid enzymes L-phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and stilbene synthase (STS), together with the final products of some of these enzymes, such as *trans*-resveratrol and total anthocyanin, and their involvement in antioxidant activity. The results, using previously isolated partial cDNAs, indicated that storage at 0 °C for 3 days increased *VcPAL*, *VcSTS* and *VcCHS* mRNA levels in the skin of non-treated grapes, decreasing slightly thereafter. In contrast, the accumulation of these transcripts was lower in the skin of grapes after 3 days of CO_2 treatment, and was undetectable when treated fruit were transferred to air. Low temperature modulated total anthocyanin levels and antioxidant capacity in non-treated grapes, and reduced the *trans*-resveratrol content in both treated and non-treated grapes, although the decrease was higher in CO_2-treated grapes. The overall results indicated that CO_2-treated grapes could be less sensitive to temperature shifts during the first stages of storage at 0 °C, reducing the expression levels of phenylpropanoid genes and the accumulation of total anthocyanins and antioxidant activity.

Keywords: Table grapes; Carbon dioxide; Phenylpropanoid pathway; Low temperature

1. Introduction

Table grape is a non-climateric fruit with significant quality losses during postharvest storage and marketing. These losses normally result from stem and pedicel desiccation, weight loss and softening of berries, and fungal decay, which is largely caused by *Botrytis cinerea*. The control of *B. cinerea* during storage and transportation is traditionally achieved by the application of SO_2 (Luvisi et al., 1992). Despite its efficacy, the use of SO_2 technology is restricted in most countries due to environmental and health concerns, justifying the search for alternatives. In the last decade, some alternative strategies, using modified atmosphere packaging (MAP) and controlled atmosphere (CA), have been tried in place of chemical treatments to control the development of decay (e.g., Crisosto et al., 2002; Retamales et al., 2003; Artés-Hernández et al., 2006; Valero et al., 2006). In previous work, we have shown that high CO_2 pretreatment for 3 days reduced total decay in table grapes stored at 0 °C, thereby maintaining fruit quality (Romero et al., 2006a; Sanchez-Ballesta et al., 2006). The effectiveness of this pretreatment was not mediated by the induction of gene expression of stilbene and the pathogenesis-related protein (PRs) when the incidence of gray mold was evident in non-treated grapes (Romero et al., 2006a; Sanchez-Ballesta et al., 2006). There are no studies showing the effect of a 3 day CO_2 pretreatment in the first stages of grape storage at 0 °C on the modulation of defense responses before the appearance of natural decay. Such studies are needed to gain a complete understanding of the mechanisms involved in such a treatment.

Phenolic compounds are a group of important secondary plant metabolites which are implicated in fruit quality, affecting the appearance, taste and flavour of fruit, and grapes constitute one of the major sources of phenolic compounds among different fruit species (Macheix et al., 1990). Since phenolic compounds have also been shown to be implicated in plant

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: mballesta@if.csic.es (Mª.T. Sanchez-Ballesta).
[1] These authors contributed equally to this work.

0925-5214/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.postharvbio.2007.04.001

resistance to different biotic and abiotic stresses (Dixon and Pavia, 1995), the study of their biosynthesis and regulation in fruit has attracted much attention. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the first committed step in the phenylpropanoid pathway, which produces a variety of phenolic compounds with structural and defense-related functions, including anthocyanins, flavonoid pigments, furanocoumarins, phytoalexins, lignin and wound phenolic esters (Hahlbrock and Scheel, 1989; Dixon and Pavia, 1995). Phytoalexins from *Vitis* species seem to constitute a rather restricted group of molecules belonging to the stilbene family (Langcake and Pryce, 1977), the skeleton of which is based on the *trans*-resveratrol structure. *Trans*-resveratrol synthesis is catalyzed by stilbene synthase (STS), which is closely related to chalcone synthase (CHS), the key enzyme of flavonoid-type compounds like anthocyanins. Both enzymes catalyze common condensation reactions of 4-coumaroyl-CoA with three malonyl-CoA units but with different cyclization reactions.

The activation of phenylpropanoid metabolism may play a role in the development of protective barriers in stress-damaged cells. For example, transgenic plants with suppressed levels of PAL exhibit spontaneous necrosis (Tamagnone et al., 1998), or more rapid and extensive lesion development than do wild-type plants after pathogen infection (Maher et al., 1994), suggesting that inhibition of phenylpropanoid biosynthesis might compromise plant health. The accumulation of *PAL*, *STS* and *CHS* mRNAs has been described in response to biotic and abiotic stresses (Leyva et al., 1995; Gläßgen et al., 1998; Brehm et al., 1999; Preisig-Müller et al., 1999; Sanchez-Ballesta et al., 2000; Franca et al., 2001; Versari et al., 2001). The coordinated accumulation of *PAL* and *CHS* mRNAs suggests that their response to such diverse stimuli may be regulated through a common controlling mechanism (Loake et al., 1991).

The aim of the present work was to analyze phenylpropanoid gene expression in red table grapes as a marker for changes in response to low temperature (0 °C) after harvest, and also to assess how the table grapes respond to high CO_2 levels (20%) at 0 °C at the end of the pretreatment and when grapes are transferred to air before the appearance of decay. To this end we analyzed different key phenylpropanoid enzymes (PAL, STS, CHS) at the transcriptional level. We also assessed levels of the final products of the action of some of these enzymes, such as *trans*-resveratrol and total anthocyanin, and examined their implication in the antioxidant activity of the skin of treated and non-treated grapes.

2. Materials and methods

2.1. Plant material

Table grapes (*Vitis vinifera* L. cv. Cardinal) were harvested at random in Camas (Sevilla, Spain) in July 2003 (12.85% total soluble solids and 0.84% tartaric acid). After harvesting, field-packaged bunches were transported to the laboratory, where fruit were immediately forced-air precooled for 14 h at −1 °C. After cooling, the bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 °C and 95% relative humidity (RH) in two sealed neoprene containers of 1 m^3 capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container in the dark. One lot was stored under normal atmosphere (non-treated fruit) and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. After 3 days, CO_2-treated grapes were transferred to air under the same conditions as non-treated fruit for up to 6 days at 0 °C. Ten clusters were sampled after cooling at −1 °C (pre-stored grapes, 0 days) and after 3 and 6 days of storage at 0 °C, and berries from five clusters (approximately 300 g each cluster) were peeled and the skin and pulp were frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis. For analysis of quality parameters, 45 berries were randomly removed from five clusters and distributed in three replicates of 15 berries each.

2.2. Quality assessments

Berry quality assessment comprised soluble solids contents (SSC), pH, titratable acidity (TA) and colour. SSC was determined using a digital refractometer Atago PR-101 (Atago Co. Ltd., Japan) at 20 °C and expressed as a percentage. The pH of the juice was measured in a pH meter with a glass electrode. TA was determined by titration with 0.1N NaOH up to pH 8.1 and results were expressed as % tartaric acid.

Berry skin colour was measured at three different positions around the equatorial region using the Hunter Lab System and a Minolta CR200^{TM} colorimeter (Minolta Camera Co., Osaka, Japan). Results were given in Commission Internationale de l'Eclairage L^* (lightness), a^*, and b^* (CIELAB) colour space coordinates. Hue ($h°$) ($\tan^{-1} b/a$) and chroma (C^*, saturation) $[(a^2 + b^2)^{1/2}]$ were calculated from a^* and b^*.

For flesh and skin firmness, grapes were tested on an Instron model 4501 (High Wycombe, UK) fitted with different cylindrical, flat-surfaced plungers, depending on the mechanical test. Skin rupture force was measured on the equatorial side of the berry using a 1 mm diameter plunger, and was determined by measuring the peak force required for the probe to penetrate the skin. Compressive firmness was measured separately on berries of approximately similar size, as the force required to compress the berry using a 35-mm diameter plunger and full-scale load of 100 N at a crosshead speed of 50 mm min^{-1}, until the berry began to release juice. Firmness was expressed in newtons (N).

2.3. Northern blot hybridization

Total RNA was extracted from the skin and pulp of grapes according to the method of Salzman et al. (1999). Samples of denatured total RNA (10 μg) were separated on 1.2% (w/v) agarose-formaldehyde gels. RNAs were transferred to Hybond-N membrane (USB, Amersham) using 20× SSC and cross-linked using a UV Stratalinker 800 (Stratagene La Jolla, CA). Equal loading was confirmed by ethidium bromide staining and by membrane staining with methylene blue.

As probes we used *PAL* (GenBank accession no. DQ887093), *CHS* (GenBank accession no. DQ887094) and *STS* (GenBank no. DQ235274) partial DNAs, previously isolated (Sanchez-

Ballesta et al., 2006; Romero et al., 2007). Probes were random-primer labelled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 °C in 7% sodium dodecyl sulphate, 0.33M phosphate buffer, pH 7.2, and 1 mM EDTA, then washed twice in 2× SSC, 0.1% SDS at room temperature and twice in 0.1× SSC, 0.1% SDS at 65 °C and then exposed to Kodak X-Omat SX film at −80 °C. Autoradiographs were digitally scanned and band densities quantified by image densitometry using Scion Image software (Scion Corporation, Frederick, MD). A value of 100% was assigned to the maximum optical density value achieved in each northern blot and the rest of optical densities were normalized to the maximum value and expressed as percentage of relative accumulation (RA).

2.4. Resveratrol content: laser analytical technique

Skin samples (0.5 g) homogenized in liquid nitrogen were extracted four times with 0.5 mL methanol at room temperature for 10 min with ultra-sonication. The extracts were centrifuged, filtered and protected from light to avoid *cis*-isomerization of stilbenes. The resveratrol content was measured as described by Orea et al. (2001) based on the combination of laser desorption (LD) with resonance-enhanced multi-photon ionization (REMPI) coupled with time-of-flight mass spectrometry (TOF-MS) detection. The results were expressed as microgram per gram fresh weight.

2.5. Analysis of total anthocyanin content

Total anthocyanin content was determined by the pH differential method as reported by Wrolstad (1976). Briefly, skin samples (0.5 g) homogenized in liquid nitrogen were extracted with 0.01% HCl in 0.5 mL methanol using ultra-sonication with cold water, four times for 10 min each. The extracts were centrifuged and the supernatants were removed and filtered. Absorbance was measured in a UV–vis spectrophotometer (UV-VIS 1601 Shimadzu) at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5 using $A = [(A_{510} - A_{700})\mathrm{pH}_{1.0} - [(A_{510} - A_{700})\mathrm{pH}_{4.5}]$ with a molar extinction coefficient of malvidin-3-glucoside of 28,000 L mol^{-1} cm^{-1}. The results were expressed as mg of malvidin-3-glucoside equivalents per gram fresh weight.

2.6. Radical cation ABTS scavenging capacity

Extraction of skin samples was performed in the same way as anthocyanin extraction described above. The radical cation 2,2′-azinobis(3-ethylbenzothiazoline 6-sulphonate) ($ABTS^+$) scavenging capacity was measured as described by Rice-Evans et al. (1996), where $ABTS^+$ is oxidized with potassium persulfate. Trolox (Hoffman-La Roche) (6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid) (2.5 mM) prepared in ethanol was used as an antioxidant standard and for the calculation of scavenging capacity of grape skin extracts as trolox equivalent. The scavenging activity of grape skin extracts was calculated as millimolar TE per gram sample.

2.7. Statistical analyses

Experimental data are the mean ± S.E. of three replicate samples of the determination for each sample. A variance analysis (one-way ANOVA) using the Fisher's least significant difference (LSD) test (Statgraphics 5.1 Plus program, STSC, Rockville, MD) was performed to determine if the *trans*-resveratrol, total anthocyanin content and antioxidant activity induced in CO_2-treated and non-treated grapes stored at 0 °C showed significant differences ($P \leq 0.05$).

3. Results

3.1. Quality of bunches of table grapes stored at 0 °C at the end of CO_2 treatment and after transferring to air

Storage at 0 °C for 3 days increased the SSC content in non-treated grapes, whereas in the 3 day CO_2-treated grapes the increase was smaller. The CO_2 treatment also restrained the decrease in TA values observed in non-treated grapes after 3 days. The pH of non-treated grapes increased after 3 days at 0 °C, but this increase was smaller in 3 day CO_2-treated ones (Table 1). At 0 °C, when 3 day-treated grapes were transferred to air the SSC, TA and pH values were similar to those observed in non-treated grapes. The external colour of the berries showed no browning in CO_2-treated fruit, and there were no significant differences between the L^*, C^* and Hue values of treated and non-treated grapes stored for 6 days at 0 °C. The combination of storage at 0 °C and CO_2 treatment did not affect skin toughness or fruit firmness compared to storage at 0 °C in air, and there were no significant differences in the puncture and compressive forces. After 6 days at 0 °C, both treated and non-treated grapes did not show any total decay (data not shown).

3.2. Phenylpropanoid gene expression in table grapes stored at 0 °C at the end of CO_2 treatment and after transfer to air

To investigate whether storage at low temperature and/or 3 days of high CO_2 treatment may modulate the pattern of phenylpropanoid gene expression in grapes, total mRNA prepared from the skin and pulp of CO_2-treated and non-treated grapes stored at 0 °C was analyzed by northern hybridization (Fig. 1).

The partial *VcPAL*, *VcSTS* and *VcCHS* probes were previously isolated (Sanchez-Ballesta et al., 2006; Romero et al., 2007). The deduced PAL amino acid sequence showed 35% identity with a *V. vinifera* PAL sequence from the cultivar 'Cabernet Sauvignon' (GenBank accession no. BAA31258) and 92% identity with a PAL from 'Lambrusco' (GenBank accession no. X75967). Comparison of the partial deduced CHS and STS amino acid sequences revealed that they were highly homologous (>90% identity) to different CHS and STS sequences from *V. vinifera*. Northern analysis revealed no *PAL*, *STS* and *CHS* gene expression in the pulp. Storage of grapes at 0 °C induced accumulation of *PAL*, *STS* and *CHS* in the skin of non-treated grapes after 3 days, decreasing slightly after 6 days (Fig. 1). In the skin of 3 day CO_2-treated grapes the increase in the

Table 1
Total soluble solids content (SSC), titratable acidity (TA), pH, colour parameters (L^*, C^* and hue), compressive and puncture firmness of grapes cv. Cardinal treated with 20% CO_2 and stored for up to 6 days at 0 °C

Quality parameters	0 day	3 day air 0 °C	3 day CO_2 0 °C	6 day air 0 °C	3 day CO_2 + 3 day air 0 °C
SSC (%)	12.85a	14.66c	13.23a	14.55c	13.75b
TA (% tartaric acid)	0.843c	0.74a	0.82c	0.8b	0.79b
pH	3.33a	3.58d	3.49c	3.44b	3.44b
L^*	36.16a	34.63a	36.48a	32.54a	33.27a
C^*	13.48a	10.03a	10.1a	9.54a	9.58a
Hue (angle)	349a	345a	345a	350a	363a
Compressive firmness (N)	21.18a	19.15a	19.15a	19.54a	18.76a
Puncture firmness (N)	0.89a	0.89a	0.95a	0.91a	0.81a

Mean in the same row with different letters (a–d) denote a statistically significant differences (LSD = 95%).

accumulation of these transcripts was lower and was practically undetectable after 6 days of cold storage.

3.3. Accumulation of trans-resveratrol, total anthocyanin and antioxidant activity in table grapes stored at 0 °C at the end of CO_2 treatment and after transfer to air

The *trans*-resveratrol content decreased significantly in the skin of non-treated grapes after 3 days at 0 °C. As occurred with *STS* gene expression, the 3 day CO_2 treatment reduced the resveratrol content 1.7 times more than in non-treated grapes stored at 0 °C. The reduction of resveratrol content induced by high CO_2 levels was also maintained when treated fruit were transferred to air at 0 °C, with values 1.5 times lower than in non-treated grapes (Fig. 2).

Total anthocyanin levels were 1.63 times greater in non-treated grapes after 3 days of storage at 0 °C than in pre-stored

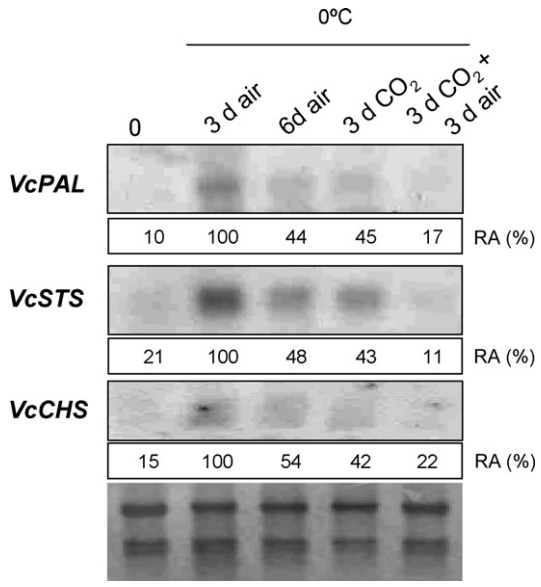


Fig. 1. Effect of high CO_2 pretreatment on *VcPAL*, *VcSTS* and *VcCHS* mRNA accumulation in the skin of table grapes cv. Cardinal stored at 0 °C. Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the partial probes. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical densities values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining.

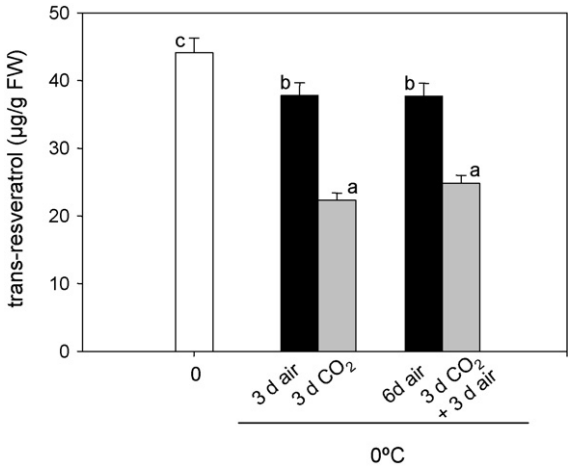


Fig. 2. Changes in *trans*-resveratrol content in the skin of non-treated and CO_2-treated table grapes cv. Cardinal stored at 0 °C. Values are the means of three replicates ± S.E. Values labeled with the same letter are not different at the 5% significance level.

grapes (0 days), whereas they were only 1.2 times higher in 3 day CO_2-treated grapes. However, when treated fruit were transferred to air at 0 °C, anthocyanin levels were significantly higher than in non-treated grapes stored for 6 days at 0 °C (Fig. 3A).

Antioxidant activity of the skin of pre-stored grapes (0 days) was 25 mM TE/g of FW. Whereas in non-treated grapes stored at 0 °C for 3 days antioxidant activity was 1.1 times higher than in pre-stored grapes, in 3 day CO_2-treated grapes the antioxidant activity decreased, reaching values 1.5 times lower than in non-treated fruit (Fig. 3B). After 6 days of storage at 0 °C, antioxidant activity significantly increased in non-treated grapes whereas similar values were quantified in 3 day CO_2-treated fruit transferred to air.

4. Discussion

It is widely accepted that low temperature storage is one of the most effective technologies to extend postharvest life, but there are no references to studies on the responses of table grapes to the first stage at low temperature. SSC and TA are important quality factors determining table grape acceptability to consumers. In general, it has been reported that SSC, TA and pH values remain fairly constant in different grape varieties dur-

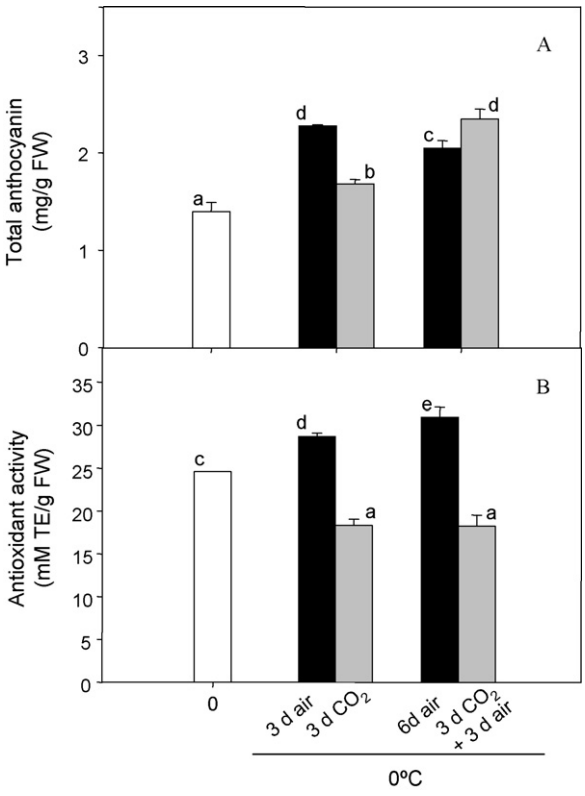


Fig. 3. Changes in total anthocyanin levels (A) and antioxidant activity (B) of non-treated and CO_2-treated table grapes cv. Cardinal stored at 0 °C. Values are the means of three replicates ±S.E. Values labeled with the same letter are not different at the 5% significance level.

ing long-term cold storage under different high CO_2 conditions (Crisosto et al., 2002; Artés-Hernández et al., 2006; Sanchez-Ballesta et al., 2006). The application of CO_2 pretreatment for 3 days at 0 °C, caused a slight increase in SSC and pH compared to those observed in pre-stored fruit. In non-treated fruit, storage at low temperature for 3 days caused a more important increase in the SSC and pH values and a decrease in TA (Table 1). After 6 days of storage, however, these differences in the quality parameters of treated and non-treated grapes were less marked. In 'Flame' table grapes, Martínez-Romero et al. (2003) observed an increase in the SSC content of about 2.5 °Brix after 4 days at 1 °C, which was about 1 °Brix when grapes were stored under MAP conditions. On the other hand, no significant changes were observed in skin colour parameters or fruit firmness between treated and non-treated grapes stored at 0 °C for up to 6 days (Table 1).

Storage for 3 days at 0 °C sharply increased the abundance of *PAL*, *STS* and *CHS* transcripts in the skin of non-treated grapes, decreasing slightly after 6 days (Fig. 1). This increase in mRNA levels in response to low temperature storage was lower after 3 days of CO_2 treatment at 0 °C and undetectable when treated fruit were transferred to air at the same temperature (Fig. 1). In photosynthetically active tissue, such as leaves of chilling tolerant *Arabidopsis thaliana*, *PAL* and *CHS* mRNA accumulate at low temperature in a light-dependent manner (Leyva et al., 1995). In the case of fruit, whereas the increase in apple PAL activity in response to different storage temperatures has been shown to be light dependent (Tan, 1980), in citrus tissues induction of *PAL* transcription and PAL activity is light independent (Sanchez-Ballesta et al., 2000). Our results in darkness indicate that a low temperature, such as 0 °C can by itself induce this phenylpropanoid gene expression in grapes. Although *V. vinifera* is tolerant to chilling, this activation of phenylpropanoid gene expression in the first stage of storage could be related to the perception by the fruit of a change in temperature, which could be less noticeable in CO_2-treated grapes. Christie et al. (1994) reported that the genes of the anthocyanin biosynthetic pathway, such as *PAL* and *CHS* can be considered 'cor' (Cold-Regulation) genes. The differences observed in phenylpropanoid gene expression between CO_2-treated and non-treated grapes could be consequence of a different perception of temperature shift. In this respect, an increasing number of studies have shown the existence of cross-adaptation in plants, so that exposure to a moderate stress not only induces resistance to this kind of severe stress but can also improve tolerance to other stresses (Bowler and Fluhr, 2000; Wang et al., 2003). In consequence, the application of high CO_2 levels (20%) in table grapes might improve tolerance to temperature shift.

Expression of *STS* genes is often induced in response to biotic and abiotic stresses, such as pathogen infection (Preisig-Müller et al., 1999), ozone (Brehm et al., 1999) and UV-light (Versari et al., 2001). To our knowledge, however, this is the first study showing regulation of *STS* gene expression by a temperature as low as 0 °C in grapes. The fact that after storage at 0 °C, the *STS* mRNA levels increased in treated and non-treated grapes while *trans*-resveratrol content decreased could indicate that low temperature affects post-transcriptional events. Furthermore, it is possible that the decrease was the result of subsequent oxidative coupling of *trans*-resveratrol producing several oligomers, such as ε and α-viniferins (Langcake and Pryce, 1977). In *Rehmannia glutinosa*, a perennial medicinal plant, resveratrol content has been found to decrease under low temperature (15 °C) treatment (Chung et al., 2006). Nevertheless, the fact that the reduction of *trans*-resveratrol content was greater in CO_2-treated than in non-treated grapes indicates a specific fruit response to low temperature and a different metabolic situation in the CO_2-treated grapes.

The induction of *PAL* and *CHS* mRNA accumulation was accompanied by an increase in total anthocyanin content, except in the case of 3 days CO_2 + 3 days air (Fig. 3A). In red 'Cardinal' grapes, a sharp increase in total anthocyanin content was observed in non-treated grapes after 3 days at 0 °C, decreasing thereafter. It is already known that anthocyanin synthesis continues after harvest and also during long-term cold storage. Mori et al. (2005) demonstrated that low night temperatures markedly increased anthocyanin synthesis in grape berries through the regulation of PAL activity and *CHS* gene expression. Veazie and Collins (2002) reported that total monomeric anthocyanin in 'Navaho' blackberries held in CA storage at 2 °C increased in the first 3 days and decreased thereafter. In contrast, high CO_2 applied as CA or MAP inhibited the postharvest increase of anthocyanin concentrations in strawberries and cherries (Gil et al., 1997; Remon et al., 2004). However, in 3 day CO_2-treated grapes the increase in total anthocyanin was significantly

smaller; they increased when grapes were transferred to air, reaching values similar to those observed in non-treated grapes after 3 days. In both cases, the increase in total anthocyanin levels was not accompanied by changes in the colour parameters measured, such as L^*, C^* and hue angle, which remained similar to those observed in pre-stored grapes.

The increase of total anthocyanin content in non-treated grapes was paralleled by an increase in antioxidant activity during low temperature storage (Fig. 3A and B). By contrast, while total anthocyanin content increased slightly in CO_2-treated grapes, there was a sharp decrease in antioxidant activity. Environmental stresses, such as low temperature induce free radicals in plants, and antioxidant activity has been found to be as much as 1.77 times higher in tomatoes stored at 5 °C than in those stored at 12 °C (Javanmardi and Kubota, 2006). Likewise, biotic and abiotic stresses increase phenolic metabolism and antioxidant capacity in spinach (Howard et al., 2002). On the other hand, no changes in antioxidant activity were detected in apples stored at low temperature under CA conditions (van der Sluis et al., 2001), but there was a decrease in antioxidant activity of samples of fresh-cut spinach stored at 10 °C under MAP (Gil et al., 1999).

The overall results suggest that table grapes, classified as chilling tolerant fruit, could be sensitive to temperature shifts during the first stage of storage at 0 °C. In non-treated grapes phenylpropanoid gene expression, total anthocyanin accumulation and antioxidant activity are activated, whereas the application of high CO_2 treatment reduces these responses. Likewise, the fact that the accumulation of some defense compounds like resveratrol was lower in CO_2-treated grapes could indicate that this treatment results in the activation of mechanisms to induce resistance in table grapes stored at low temperature. However, further analyses are needed to identify mechanisms associated to the response of table grapes to 0 °C in the first stage of storage and to determine whether high CO_2 levels could modulate them.

Acknowledgements

This work was supported by research grants (AGL2005-04502/ALI and AGL2002-02308) from the CICYT (Spain) and CTQ2004-3468 from the MEC of Spain. M.T.S.-B. and I.R. were supported by a postdoctoral I3P contract and postgraduate I3P fellowship from the CSIC, respectively. The authors are grateful to Frutaria SAT for providing tables grapes and to Agrotecnica for industrial assistance.

References

Artés-Hernández, F., Tomás-Barberán, F.A., Artés, F., 2006. Modified atmosphere packaging preserves quality of SO_2-free 'Superior seedless' table grapes. Postharvest Biol. Technol. 39, 146–154.

Bowler, C., Fluhr, R., 2000. The role of calcium and activated oxygen as signals for controlling cross-adaptation. Trends Plant Sci. 5, 241–246.

Brehm, I., Preisig-Müller, R., Kindl, H., 1999. Grapevine protoplasts as a transient expression system for comparison of stilbene synthase genes containing cGMP-responsive promoter elements. Z. Naturforsch. (C) 54, 220–229.

Christie, P.J., Alfenito, M.R., Walbot, V., 1994. Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta 194, 541–549.

Chung, I.M., Kim, J.J., Lim, J.D., Yu, C.Y., Kim, S.H., Hahn, S.J., 2006. Comparison of resveratrol, SOD activity, phenolic compounds and free amino acids in Rehmannia glutinosa under temperature and water stress. Environ. Exp. Bot. 56, 44–53.

Crisosto, C.H., Garner, D., Crisosto, G., 2002. Carbon dioxide-enriched atmospheres during cold storage limit losses from *Botrytis* but accelerate rachis browning of 'Redglobe' table grapes. Postharvest Biol. Technol. 26, 181–189.

Dixon, R.A., Pavia, N.L., 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097.

Franca, S.C., Roberto, P.G., Marins, M.A., Puga, R.D., Rodrigues, A., Pereira, J.O., 2001. Biosynthesis of secondary metabolites in sugarcane. Genet. Mol. Biol. 24, 243–250.

Gil, M.I., Ferreres, F., Tomas-Barberan, F.A., 1999. Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. J. Agric. Food Chem. 47, 2213–2217.

Gil, M.I., Holcroft, D.M., Kader, A.A., 1997. Changes in strawberry anthocyanins and other polyphenols in response to carbon dioxide treatments. J. Agric. Food Chem. 45, 1662–1667.

Gläβgen, W.E., Rose, A., Madlung, J., Koch, W., Gleitz, J., Seitz, H.U., 1998. Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. Planta 204, 490–498.

Hahlbrock, K., Scheel, D., 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 17, 425–429.

Howard, L.R., Pandjaitan, N., Morelock, T., Gil, M.I., 2002. Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. J. Agric. Food Chem. 50, 5891–5896.

Javanmardi, J., Kubota, C., 2006. Variation of lycopene, antioxidant activity, total soluble solids and weight loss of tomato during postharvest storage. Postharvest Biol. Technol. 41, 151–155.

Langcake, P., Pryce, R.J., 1977. A new class of phytoalexins from grapevines. Experientia 33, 151–152.

Leyva, A., Jarillo, J.A., Salinas, J., Martinez-Zapater, J.M., 1995. Low-temperature induces the accumulation of *phenylalanine ammonia-lyase* and *chalcone synthase* mRNAs of *Arabidopsis thaliana* in a light-dependent manner. Plant Physiol. 108, 39–46.

Loake, G.J., Choudhary, A.D., Harrison, M.J., Mavandad, M., Lamb, C.J., Dixon, R.A., 1991. Phenylpropanoid pathway intermediates regulate transient expression of a *chalcone synthase* gene promoter. Plant Cell 3, 829–840.

Luvisi, D.A., Shorey, H., Smilanick, J., Thompson, J., Gump, B., Knutson, J., 1992. Sulfur Dioxide Fumigation of Table Grapes. University of California, DANR, Bulletin, p. 1932.

Macheix, J.J., Fleuriet, A., Billot, J., 1990. The main phenolics of fruits. In: Fruit Phenolics. CRC Press, Boca Raton, FL, pp. 1–98.

Maher, E.A., Bate, N.J., Ni, W., Elkind, Y., Dixon, R.A., Lamb, C.J., 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels pf preformed phenylpropanoid products. Proc. Natl. Acad. Sci. U.S.A. 91, 7802–7806.

Martínez-Romero, D., Guillén, F., Castillo, S., Valero, D., Serrano, M., 2003. Modified atmosphere packaging maintains quality of table grapes. J. Food Sci. 68, 1838–1843.

Mori, K., Sugaya, S., Gemma, H., 2005. Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. Sci. Hortic. 105, 319–330.

Orea, J.M., Montero, C., Jiménez, J.B., González-Ureña, A., 2001. Analysis of trans-resveratrol by laser desorption coupled with resonant ionization spectrometry. Application to trans-resveratrol content in vine leaves and grape skin. Anal. Chem. 73, 5921–5959.

Preisig-Müller, R., Schwekendiek, A., Brehm, I., Reif, H.J., Kindl, H., 1999. Characterization of a pine multigene family containing elicitor-responsive stilbene synthase genes. Plant Mol. Biol. 39, 221–229.

Remon, S., Ferrer, A., López-Buesa, P., Oria, R., 2004. Atmosphere composition effects on Burlat cherry colour during cold storage. J. Sci. Food Agric. 84, 140–146.

Retamales, J., Defilippi, B.G., Arias, M., Castillo, P., Manríquez, D., 2003. High-CO_2 controlled atmospheres reduce decay incidence in Thompson Seedless and Red Globe table grapes. Postharvest Biol. Technol. 29, 177–182.

Rice-Evans, C.A., Miller, N.J., Paganda, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Rad. Biol. Med. 20, 933–956.

Romero, I., Sanchez-Ballesta, M.T., Maldonado, R., Escribano, M.I., Merodio, C., 2006a. Expression of class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO_2 pretreatment. Postharvest Biol. Technol. 41, 9–15.

Romero, I., Sanchez-Ballesta, M.T., Maldonado, R., Escribano, M.I., Merodio, C., 2007. Molecular regulation of anthocyanin biosynthesis, ascorbate peroxidase and antioxidant activity in the skin of CO_2-treated table grape stored at low temperature. J. Plant Physiol., in press.

Salzman, R.A., Fujita, T., Zhu-Salzman, K., Hasegawa, P.M., Bressan, R.A., 1999. An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol. Biol. Rep. 17, 11–17.

Sanchez-Ballesta, M.T., Jiménez, J.B., Romero, I., Orea, J.M., Maldonado, R., González-Ureña, A., Escribano, M.I., Merodio, C., 2006. Effect of high CO_2 pretreatment on quality, fungal decay and molecular regulation of stilbene phytoalexin biosynthesis in stored table grape. Postharvest Biol. Technol. 42, 209–216.

Sanchez-Ballesta, M.T., Lafuente, M.T., Zacarias, L., Granell, A., 2000. Involvement of phenylalanine ammonia-lyase in the response of Fortune mandarin fruits to cold temperature. Physiol. Plant 108, 382–389.

Tamagnone, L., Merida, A., Stacey, N., Paskitt, K., Parr, A., Chang, C.F., Lynn, D., Dow, J.M., Roberts, K., Martin, C., 1998. Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. Plant Cell 10, 1801–18016.

Tan, S.C., 1980. Phenylalanine ammonia-lyase and phenylalanine ammonia lyase inactivation system effects of light, temperature and mineral deficiencies. Aust. J. Plant Physiol. 7, 159–168.

Valero, D., Valverde, J.M., Martinez-Romero, D., Guillen, F., Castillo, S., Serrano, M., 2006. The combination of modified atmosphere packaging with eugenol or thymol to maintain quality, safety and functional properties of table grapes. Postharvest Biol. Technol. 41, 317–327.

van der Sluis, A.A., Dekker, M., de Pager, A., Jongen, W.M.F., 2001. Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. J. Agric. Food Chem. 49, 3606–3613.

Veazie, P.P., Collins, J.K., 2002. Quality of erect-type blackberry fruit after short intervals of controlled atmosphere storage. Postharvest Biol. Technol. 25, 235–239.

Versari, A., Parpinello, G.P., Tornielli, G.B., Ferrarini, R., Giulivo, C., 2001. Stilbene compounds and stilbene synthase expression during ripening, wilting, and UV treatment in grape cv. Corvina. J. Agric. Food Chem. 49, 5531–5536.

Wang, W.X., Vinocur, B., Altman, A., 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218, 1–14.

Wrolstad, R.E., 1976. Colon and Pigment Analysis in Fruit Products, Corvallis. Oregon Agricultural Experimental Station Bulletin, Oregon, p. 624.

ARTÍCULO 3

Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature.

Irene Romero, M. Teresa Sanchez-Ballesta, Roberto Maldonado, M. Isabel Escribano, Carmen Merodio

Journal of Plant Physiology (*Available online 13 June 2007)*

RESUMEN

Un pretratamiento con 20 kPa CO_2+20 kPa O_2+60 kPa N_2 durante 3 días fue efectivo en mantener la calidad de la fruta y controlar el ataque por hongo de uvas de mesa (*Vitis vinifera* cv.Cardinal) almacenadas a 0ºC. En este trabajo, analizamos si el contenido en antocianos totales, el mecanismo molecular implicado en su biosíntesis y la actividad antioxidante está relacionado con el efecto beneficioso de este tratamiento gaseoso. Aislamos cDNAs parciales que codifican para enzimas implicadas en la biosíntesis de antocianos tales como L-fenilalanina amonio-liasa (PAL) y chalcona sintasa (CHS) y una enzima antioxidante como la ascorbato peroxidasa (APX). Las bajas temperaturas indujeron una acumulación de antocianos totales en la piel de uvas tratadas y no tratadas, aunque los niveles fueron menores en fruta tratada con CO_2. Por el contrario, la actividad antioxidante decreció durante el almacenamiento a 0ºC en uvas no tratadas pero no cambió en uvas tratadas con CO_2. El aumento en los niveles de expresión de los genes asociados a la biosíntesis de antocianos y del mRNA *VcAPX* observado en uvas no tratadas, no se observó en uvas tratadas con CO_2, las cuales presentaron bajos niveles de ataque por hongo. Estos resultados señalan la habilidad de las uvas tratadas con CO_2 para prevenir la generación de especies reactivas de oxígeno más que su inactivación por medio de la inducción de los sistemas de defensa estudiados.

Journal of Plant Physiology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Available online at www.sciencedirect.com
ScienceDirect

JOURNAL OF PLANT PHYSIOLOGY
www.elsevier.de/jplph

Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature

Irene Romero, M. Teresa Sanchez-Ballesta*, Roberto Maldonado, M. Isabel Escribano, Carmen Merodio

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frio, IF-CSIC, Ciudad Universitaria, E-28040 Madrid, Spain

Received 24 October 2006; received in revised form 19 December 2006; accepted 21 December 2006

KEYWORDS
Anthocyanin biosynthesis;
Ascorbate peroxidase;
Carbon dioxide;
Gene expression;
Table grapes

Summary

A pretreatment with 20 kPa CO_2+20 kPa O_2+60 kPa N_2 for 3 days proved effective in maintaining the fruit quality and controlling decay in table grapes (*Vitis vinifera* cv. Cardinal) stored at 0 °C. In the present work, we analyzed whether total anthocyanin content, the molecular mechanism implicated in their biosynthesis and antioxidant activity is related to the beneficial effect of this gaseous treatment. We isolated partial cDNAs that codified for enzymes implicated in the anthocyanin biosynthesis such as L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), and an antioxidant enzyme such as ascorbate peroxidase (APX). Low temperatures induced an accumulation of total anthocyanin content in the skin of both treated and non-treated grapes, although levels were lower in CO_2-treated fruit. By contrast, antioxidant activity decreased during storage at 0 °C in non-treated grapes but did not change in CO_2-treated grapes. The up-regulation of anthocyanin biosynthesis gene expression and *VcAPX* mRNA observed in non-treated grape is not enhanced in CO_2-treated grapes, which presented low total decay. These results point out the ability of CO_2-treated grapes to prevent the generation of reactive oxygen species rather than their inactivation by means of induction of studied defense systems.

Introduction

One of the main goals of postharvest technology in table grapes is to delay fungal attack, mainly caused by *Botrytis cinerea*, which causes extensive

Abbreviations: APX, ascorbate peroxidase; CA, controlled atmosphere; CHS, chalcone synthase; FW, fresh weight; MAP, modified atmosphere packaging; PAL, L-phenylalanine ammonia-lyase; ROS, reactive oxygen species; TE, equivalent of trolox

*Corresponding author. Tel.: +34 91 5492300; fax +34 91 5493627.
E-mail address: mballesta@if.csic.es (M. Teresa Sanchez-Ballesta).

0176-1617/$ - see front matter © 2007 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2006.12.011

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

losses during cold storage. The possibility of safe alternative control methods to assure high-quality fruit and control fungal attack is really important because postharvest chemical treatments are restricted in most countries. Among the alternative methods for controlling table grapes postharvest decay, such as modified atmosphere packaging (MAP) (Artés-Hernández et al., 2006) and controlled atmosphere (CA) (Yahia et al., 1983; Crisosto et al., 2002), high CO_2 pretreatment for shorter storage periods during postharvest handling of table grapes offers interesting possibilities. In previous works, we reported that pretreatment with 20% CO_2 and 20% O_2 for 3 days reduced fungal decay in table grapes stored at 0 °C while maintaining the fruit quality (Romero et al., 2006; Sanchez-Ballesta et al., 2006). The 3-day high CO_2 pretreatment also reduced rachis browning, which is a limitation on prolonged CA storage (Crisosto et al., 2002; Retamales et al., 2003). However, little is known about the physiological and molecular events occurring in response to the application of high CO_2 levels in table grapes. Our previous studies indicated that the efficacy of high CO_2 pretreatment is not mediated by the induction of pathogenesis-related proteins such as class I chitinase and β-1,3-glucanase (Romero et al., 2006). Moreover, the stilbene phytoalexin biosynthesis in CO_2-treated fruit was not induced (Sanchez-Ballesta et al., 2006). Stilbene synthase (STS), the enzyme leading to stilbene synthesis, is closely related to chalcone synthase (CHS), the enzyme implicated in the biosynthesis of flavonoid-type compounds like anthocyanins. CHS and STS catalyze common condensation reactions of *p*-coumaroyl-CoA and three C(2)-units from malonyl-CoA but different cyclization reactions to produce naringenin chalcone and resveratrol, respectively. Owing to the fact that the development of protection strategies of CO_2-treated grapes is not based on the induction of *STS* gene expression and resveratrol accumulation, this work focuses on the effect of high CO_2 levels on anthocyanin biosynthesis.

Anthocyanins, the main pigments in flowers and fruits, are a flavonoid subclass synthesized from hexose through the shikimate, phenylpropanoid and flavonoid pathways. The anthocyanin biosynthetic pathway is one of the well-known pathways in plants (Holton and Cornish, 1995). The first committed step is catalyzed by CHS, which condenses the metabolites malonyl-CoA and 4-coumaroyl-CoA formed essentially via the shikimate and/or malonate pathways. It has been suggested that anthocyanins function as photoprotective pigments (Li et al., 1993), and anthocyanin accumulation can reportedly be induced in many plants by biotic and abiotic stresses, such as UV-B radiation, pathogen attack or low temperature (Mancinelli, 1983; Winkel-Shirley, 2001). In view of these findings, it is possible that the efficacy of high CO_2 pretreatment in preventing fungal attack was related with the effect on the synthesis of such protective phenolic compounds. Most studies of table grapes treated with CO_2 have addressed the quantity and type of anthocyanins, but there is still a dearth of knowledge about the expression of the anthocyanin biosynthetic genes in relation to these beneficial postharvest treatments.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is the enzyme at the entry-point of the phenylpropanoid pathway, which yields a variety of phenolic compounds with structural and defense-related functions. PAL catalyzes the deamination of L-phenylalanine to form *trans*-cinnamic acid, which eventually leads to the production of *p*-coumaroyl-CoA the common substrate of STS and CHS. Owing to the nature and function of the products derived from the phenylpropanoid pathway, PAL activity and the activation of PAL under stress conditions have been considered part of a defense mechanism operating in stress-afflicted cells (Dixon and Pavia, 1995). Therefore, molecular analysis of PAL was also chosen as a mean to understand the effect of high CO_2 levels on table grapes stored at low temperatures.

Production of reactive oxygen species (ROS) during so-called ''oxidative burst'' is thought to be a central event in activation of disease resistance. In plant cells, the enhanced generation of ROS in response to both abiotic and biotic constraints has been well documented (Mehdy et al., 1996; Alscher et al., 1997). However, it is not clear whether changes in ROS generation are directly involved in the activation of plant defense response or are a mere consequence of the oxidative stress occurring in the attacked cells. Oxidative events are associated with the different *B. cinerea*–plant interactions, but their precise role in such interactions remains unclear (Grovin and Levine, 2000; Tierens et al., 2002). Hydrogen peroxide (H_2O_2) represents a crucial crossing in the mechanisms of oxidative stress, and its regulation plays an important role in cell life. Therefore, different systems are utilized by cell to balance the levels of H_2O_2. Ascorbate peroxidases (APX) (EC 1.11.1.11) utilize ascorbic acid and its specific electron to reduce H_2O_2 to water. APX genes have been isolated from some plants and their expression has been determined under fruit ripening, oxidative stress and low temperature, respectively (Kim and Chung, 1998; Yoshimura et al., 2000; Kawakami et al., 2002). In grape, there have been

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

no reports on the involvement of APX in *Botritys* attack or on the effect of a beneficial high CO_2 pretreatment in controlling decay; we therefore isolated an *APX* partial cDNA and analyzed the changes in gene expression in treated and non-treated table grapes stored at low temperatures.

In order to further our knowledge of the mode of action of high CO_2 pretreatment, we have analyzed total anthocyanin content and antioxidant capacity as they relate to the beneficial effect of the gaseous treatment. Considering the role of PAL and CHS in the anthocyanin biosynthesis, we have cloned a *VcCHS* and *VcPAL* partial cDNA clones and monitored their expression in treated and non-treated grapes during low-temperature storage. This paper also reports our findings on the relationship between *APX* regulation at transcript level and susceptibility to development of natural fungal decay at low temperature.

Materials and methods

Plant material

Table grapes (*Vitis vinifera* L. cv. 'Cardinal') were harvested at random in Camas (Sevilla, Spain) in July. Early-harvesting mature berries were used in this work (12.85% total soluble solids; 0.84% tartaric acid). After harvesting, field-packaged bunches were transported to the laboratory, where fruits were immediately forced-air precooled for 14 h at −1 °C. After cooling, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 °C and 95% relative humidity in two sealed neoprene containers of 1 m^3 capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container in the dark. One lot was stored under normal atmosphere for 33 days (non-treated fruit) and the other under a gas mixture containing 20% CO_2+20% O_2+60% N_2 (CO_2-treated fruit) for 3 days. The CO_2 concentration was maintained throughout the pretreatment experiment and was measured daily using an automated gas chromatograph system equipped with a thermal conductivity detector and Poraplot Q column (Varian Chrompack CP20033P). After 3 days, CO_2-treated grapes were transferred to air under the same conditions as non-treated fruits until the end of the storage period. Ten clusters were sampled periodically, and berries from five clusters (approx. 300 g each cluster) were peeled and the skin and pulp were frozen in liquid nitrogen, grounded to a fine powder and stored at −80 °C until analysis.

Cloning of PAL, CHS and APX partial cDNAs

Total RNA was extracted from the skin of grapes according to the method of Salzman et al. (1999). Partial cDNA clones of PAL, CHS and APX were obtained by RT-PCR. cDNA synthesis was performed with 10 μg of a mixture of total RNA from the skin of grapes stored in air and CO_2 for 12 and 33 days. The reaction was carried out in the presence of 500 ng of oligo-dT with 100 U of Reverse Transcriptase (Ecogen). PAL, CHS and APX gene DNA fragments were obtained by PCR amplification using the cDNA as template and the combination of the sense and the antisense primers. A 668 bp fragment of PAL was amplified by combining the degenerate sense primer 5′-CAATGGCTNGGCCCNCAYATHGAA-3′ and the degenerate antisense primer 5′-AARCGNATGTARCTACTGGGGACG-3′. A 634 bp fragment of CHS was amplified using a sense primer 5′-TGATTACTACTTCCGCATCACCA-3′ and the antisense primer 5′-CTTCCTTCTCTTTGACTCTCGTTGA-3′. A 539 bp fragment of APX was amplified using a degenerate sense primer 5′-GCATGGCACTCTGCTGGWAC-3′ and the degenerate antisense primer 5′-TCATCSG-CAGCRTATTTYTC-3′. PCR products were cloned into the pGEMT vector (Promega) and confirmed by sequencing.

RNA gel blot hybridization

Samples of denatured total RNA (10 μg) from the skin of grapes were fractionated and blotted as described in Sanchez-Ballesta et al. (2000). Equal loading was confirmed by ethidium bromide staining and by membrane staining with methylene blue. The DNA probe was random-primer labeled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 °C in 7% sodium dodecyl sulfate, 0.33 M phosphate buffer, pH 7.2, and 1 mM EDTA, then washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 0.1 × SSC, 0.1% SDS at 65 °C and exposed to Kodak X-Omat SX film at −80 °C. Methylene blue stained membranes and autoradiographs were digitally scanned and quantification was performed using a computer-assisted image analysis system (Scion Corporation, Frederick, MD). To ensure the accuracy of the changes in mRNA abundance and equal loading of RNA, the optical density values achieved in each Northern were normalized to the amount of 18S rRNA on the methylene blue staining. The 100% was assigned to the maximum optical density after normalization to the 18S rRNA in each Northern and the rest of optical densities were normalized to the maximum value and expressed as percentage of relative accumulation (RA).

PAL activity assay

PAL was extracted from 5 g of skin according to the method of Faragher and Chalmers (1977). The skin was ground in 25 ml of 100 mM H_3BO_3-NaOH buffer (pH 8.8) containing 25 mM 2-mercaptoethanol and 6% PVP. Afterwards, it was filtered through two layers of gauze and centrifuged for 30 min at 14,000*g*. The supernatant was saturated with 70% $(NH_4)_2SO_4$ and the pellet was sedimented by centrifuging 14,000*g* for 30 min, redissolved in the same buffer and used for the enzyme assay.

The assay was based on the method of Tanaka et al. (1974). The reagent mixture containing 2.5 ml of 100 mM borate-NaOH buffer (pH 8.8) and 0.5 ml of 40 mM

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

phenylalanine was preincubated at 30 °C for 5 min and the enzyme was added into the solution. The assay continued for 30 min at 30 °C and was stopped by adding 0.5 ml of 2 M perchloric acid. Prior to determination of OD at 280 nm, the mixture was centrifuged at 2000*g* for 15 min. The control tube included perchloric acid at the start of the assay. Specific enzyme activity was defined as nmol of cinnamic acid/h/mg of protein.

Protein concentration was measured by the method of Bradford (1976) using protein-dye reagent (Bio-Rad, Hercules, CA) and bovine serum albumin (Sigma-Aldrich, St Louis, MO) as a standard.

Analysis of total anthocyanin content

Total anthocyanin contents were determined by the pH differential method as reported by Wrolstad (1976). Briefly, skin samples (0.5 g) homogenized in liquid nitrogen were extracted with 0.01% HCl in 0.5 ml methanol using ultrasonication with cold water, four times for 10 min each. The extracts were centrifuged at 4000*g* and the supernatants were removed and filtered. Absorbance was measured in a UV–VIS spectrophotometer (UV-VIS 1601 Shimadzu) at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5 using $A = ((A_{510}-A_{700})\ pH_{1.0}-[(A_{510}-A_{700})\ pH_{4.5})$ with a molar extinction coefficient of malvidin-3-glucoside of 28,000 $L\,mol^{-1}\,cm^{-1}$. The results were expressed as milligrams of malvidin-3-glucoside equivalent per gram fresh weight (FW).

Radical cation ABTS scavenging capacity

The extraction of skin samples was performed in the same way as anthocyanin extraction described above. The radical cation 2,2′-azinobis(3-ethylbenzothiazoline 6-sulphonate) ($ABTS^+$) scavenging capacity was measured as described by Rice-Evans et al. (1996), where $ABTS^+$ is oxidized with potassium persulfate. Trolox (Hoffman-La Roche; 6-hydroxy 2,5,7,8-tetramethylchroman- 2-carboxylic acid; 2.5 mM) prepared in ethanol was used as an antioxidant standard and for the calculation of scavenging capacity of grape skin extracts as trolox equivalent. The scavenging activity of grape skins extracts was calculated as mM Trolox equivalent (TE)/g FW.

Statistical analyses

Experimental data are the mean ± SE of three replicate samples of the determination for each sample. A variance analysis (one-way ANOVA) using the Fisher's least significant difference test (Statgraphics 5.1 Plus program, STSC, Rockville, MD) was performed to determine whether the total anthocyanin content, antioxidant activity and PAL activity induced in treated and non-treated grapes stored at 0 °C showed significant differences ($p \leqslant 0.05$).

Results

Total anthocyanin content and antioxidant activity in non-treated and CO_2-treated grapes stored at 0 °C

Total anthocyanin content increased significantly after 12 days at 0 °C both in non-treated and CO_2-treated grapes although it was higher in non-treated ones (Fig. 1). Thereafter, the accumulation of total anthocyanin decreased in both treated and non-treated fruit although at the end of the storage the accumulation was higher in non-treated grapes.

In CO_2-treated grapes, antioxidant activity was higher than in non-treated fruit (Fig. 2). The antioxidant capacity in non-treated grapes decreased significantly along the storage reaching minimum by day 22 at 0 °C (Fig. 2). In CO_2-treated fruit, changes in antioxidant capacity during storage at 0 °C were not significant, remaining similar to those achieved in freshly harvested grapes.

Effect of high CO_2 levels on anthocyanin gene expression (PAL, CHS) and PAL activity during low-temperature storage of grapes

To investigate the pattern of anthocyanin gene expression in response to a 3-day CO_2 pretreatment, total mRNA prepared from the skin of CO_2-treated and non-treated grapes stored at 0 °C for up to 33 days was analyzed by northern hybridization, and PAL activity was determined.

The partial cDNA clones used as probes were obtained by RT-PCR (PAL, GenBank accession no.

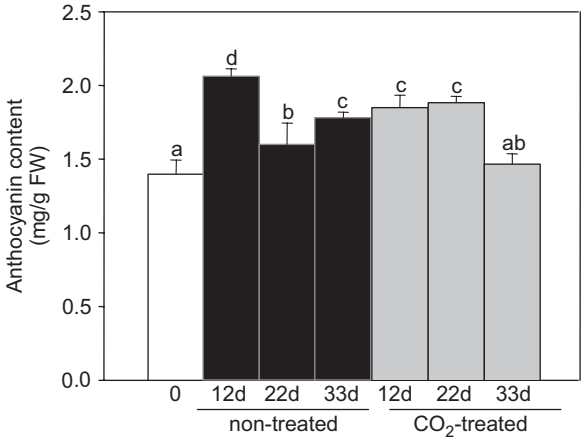


Fig. 1. Changes in total anthocyanin content in the skin of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 °C. Results are the mean of three replicate samples ± SE. Values labeled with the same letter are not different at the 5% significance level.

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

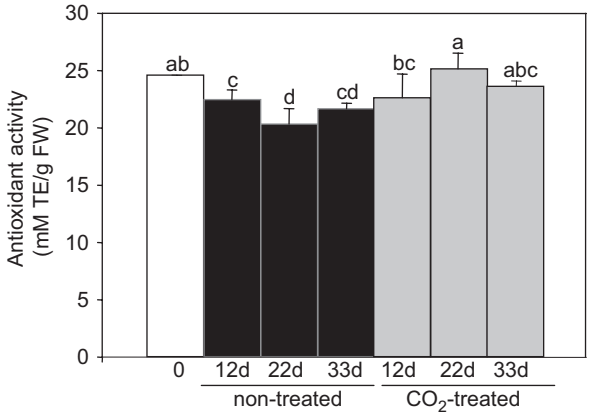


Fig. 2. Changes in antioxidant activity expressed as TE (mM/g FW) of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 °C. Results are the mean of three replicate samples ± SE. Values labeled with the same letter are not different at the 5% significance level.

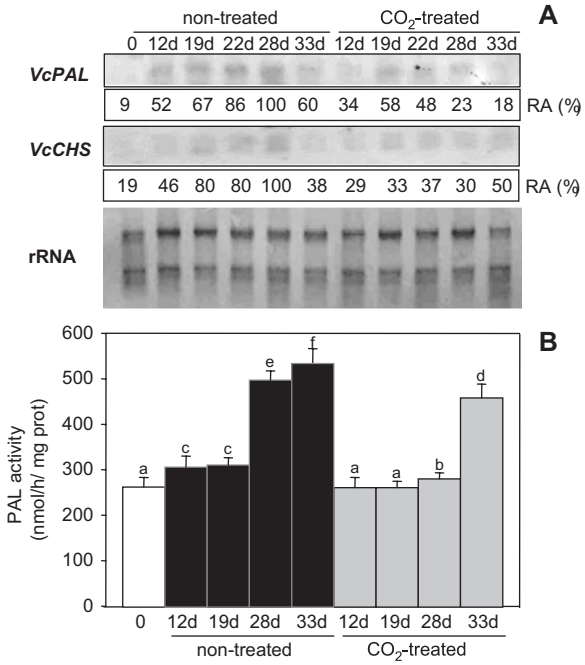


Fig. 3. Effect of high CO_2 pretreatment on *VcPAL* and *VcCHS* mRNA accumulation in the skin of 'Cardinal' table grapes stored at 0 °C (A). Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the partial *VcPAL* and *VcCHS* probes. The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. The intensity of the bands was quantified by scanning densitometry of the autoradiographs and it was normalized to the amount of 18S rRNA on the methylene blue staining. Optical density values were normalized to the maximum value and expressed as percentage of RA. (B) Time courses of PAL activity in the skin of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 °C. Data are the average of two separate experiments ($n = 6$) ± SE. Values labeled with the same letter are not different at the 5% significance level.

DQ887093 and CHS, GenBank accession no. DQ887094). The partial *VcPAL* cDNA consisted of 668 bp and encoded a polypeptide of 222 amino acids. The partial amino-acid sequence shared about 93% identity with PAL sequences from lemon, mandarin fruit and aspen. As regards *V. vinifera* PAL sequences, the deduced PAL amino-acid sequence showed 35% identity with a PAL sequence from the cultivar Cabernet Sauvignon (GenBank accession no. BAA31258) and 92% identity with a PAL from Lambrusco (GenBank accession no. X75967). The partial *VcCHS* cDNA consisted of 634 bp and encoded a polypeptide of 211 amino acids. A comparison of the partial deduced amino-acid sequence revealed that it was highly homologous (98% identity) to different CHS sequences from *V. vinifera* (Cv. Cabernet Sauvignon, CHS2 GenBank accession no AB66275, psCH4 GenBank accession no. AB015872; Cv. Lambrusco Foglia Frastagliata, CHS GenBank accession no. X75969).

Storage of grapes at 0 °C induced the accumulation of *PAL* and *CHS* mRNA levels in the skin of non-treated and CO_2-treated grapes (Fig. 3A). However, both the *VcPAL* and the *VcCHS* mRNA levels in non-treated grapes were higher than in CO_2-treated fruit. In non-treated grapes, a sharp increase was observed in the levels of *PAL* mRNA after 12 days at 0 °C reaching the maximum by day 28 and decreasing thereafter. The accumulation of *CHS* transcript, on the other hand, was slow but progressive, reaching maximum after 28 days at 0 °C like the *PAL* mRNA and decreasing in the levels by day 33. In CO_2-treated grapes, the levels of *VcCHS* transcripts increased after 19 days and did not change until the end of the storage. Also, the *VcPAL* transcript levels increased after 19 days and decreased after 33 days. Transcript levels remained lower than that in non-treated grapes throughout storage. As probes were used the partial cDNAs isolated showed high homology with different *PAL* and *CHS* sequences, indicating that global expression of different isogenes could be measured. Northern analysis revealed that *PAL* and *CHS* gene expression was not detected in the pulp of either treated or non-treated grapes (data not shown).

PAL activity increased slightly in non-treated grapes after 12 days at 0 °C, showing a sharp increase by day 28, paralleled to the higher accumulation in *PAL* transcript and reaching maximum after 33 days (Fig. 3B). PAL activity was significantly lower in CO_2-treated grapes than that

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

in non-treated fruit reaching maximum by day 33, although this maximum was lower than that recorded in non-treated grapes, the same pattern as in PAL mRNA accumulation.

Effect of high CO_2 levels on APX gene expression during low-temperature storage of grapes

A partial *APX* cDNA clone was obtained by RT-PCR using as template a mixture of cDNAs synthesized from total RNA extracted from the skin of CO_2-treated and non-treated grapes stored at 0 °C. A cDNA fragment of 539 bp was cloned and named *VcAPX* (GenBank accession no. DQ887095). The deduced sequence of 179 amino acid presented the heme-binding domain that share all APX proteins, and it showed 82–98% similarity with amino-acid sequences of APX genes accessed in GenBank, being the greatest similarity to cytosolic APX sequences.

VcAPX transcript levels increased in non-treated grapes after 12 days, reaching maximum by day 28 and decreasing thereafter (Fig. 4). In CO_2-treated grapes, on the other hand, the increase in the accumulation of the transcript was smaller and did not change during storage at 0 °C.

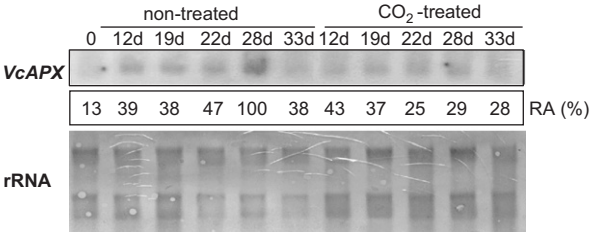


Fig. 4. Effect of high CO_2 pretreatment on *VcAPX* mRNA accumulation in the skin of 'Cardinal' table grapes stored at 0 °C. Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the partial *VcAPX* probe. The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. The intensity of the bands was quantified by scanning densitometry of the autoradiographs, and it was normalized to the amount of 18S rRNA on the methylene blue staining. Optical density values were normalized to the maximum value and expressed as percentage of RA.

Discussion

The use of high CO_2 concentrations to enhance table grapes quality in place of SO_2 fumigation has been a focus of interest in recent years (Crisosto et al., 2002; Retamales et al., 2003; Artés-Hernández et al., 2006; Romero et al., 2006; Sanchez-Ballesta et al., 2006). However, there is little understanding of its possible mode of action, although many authors have reported that high CO_2 treatments maintain grape quality by retarding fungal attack, mainly caused by *B. cinerea*. In previous works, we observed that a 3-day high CO_2 pretreatment significantly reduced natural fungal decay in table grapes cv. Cardinal stored at 0 °C for up to 33 days, but that this beneficial effect was not mediated by the induction of PRs and STS gene expression (Romero et al., 2006; Sanchez-Ballesta et al., 2006). STS and CHS, the enzymes of resveratrol and anthocyanin biosynthesis respectively, each catalyze the formation of a tetraketide intermediate from a CoA-tethered phenylpropanoid starter and three molecules of malonyl-CoA, but use different cyclization mechanisms to produce the end products. Likewise, PAL catalyzes the first step of the multi-branched general phenylpropanoid pathway, which supplies substrates for biosynthesis of different phenolic compounds such as the end products of the reactions catalyzed by the STS and CHS enzymes. Since we observed that high CO_2 pretreatment did not modulate *STS* gene expression to control fungal decay, a new area of research is open to further investigate the molecular mechanisms implicated in the mode of action of high CO_2 concentrations in table grapes by analyzing anthocyanin biosynthesis gene expression.

The total anthocyanin content increased in both treated and non-treated grapes after 12 days of storage at 0 °C, decreasing thereafter, reaching in CO_2-treated grapes levels similar to those observed in freshly harvested grapes (Fig. 1) after 33 days. This trend in anthocyanin levels match with a decrease in L^*, Croma and hue angle color parameters, although no significant changes between treated and non-treated grapes were quantified (data not shown). Mori et al. (2005) demonstrated that environmental factors affect anthocyanin synthesis in grape berries through the regulation of PAL activity and *CHS* gene expression. They showed that anthocyanin synthesis, PAL activity and *CHS3* transcript levels in Darkridge grapes were markedly higher in berries grown under low night temperatures. It is already known that anthocyanin synthesis continues after harvesting and also in low-temperature storage (Kalt et al., 1999). The application of high CO_2 as a pretreatment in table grapes causes an increase in the total anthocyanin content during storage at 0 °C, although this increase was lower than that in non-treated grapes.

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

By contrast, it has been reported that CA or MAP treatments inhibit postharvest increase of anthocyanin concentrations in different fruits (Gil et al., 1997; Remon et al., 2004). Nevertheless, Veazie and Collins (2002) reported that total monomeric anthocyanin in Navaho blackberries held in CA storage at 2 °C increased in the first 3 days and decreased thereafter.

The increase of total anthocyanin content in treated and non-treated grapes did not produce any increase in antioxidant activity during low-temperature storage (Fig. 2). However, it is interesting to note that although antioxidant activity in the treated grapes stored at low temperature was similar to that of freshly harvested fruit, it decreased in the non-treated fruit. No changes in antioxidant activity were detected in apples stored at low temperatures and under CA conditions (van der Sluis et al., 2001), but there was a decrease in antioxidant activity of samples of fresh-cut spinach stored under MAP at 10 °C (Gil et al., 1999).

To analyze anthocyanin biosynthesis gene expression, we have isolated partial cDNAs encoding PAL and CHS from the skin of table grapes. The predicted VcPAL amino-acid sequence shared significant levels of identity with the published sequence from *V. vinifera* Cv. Lambrusco (Sparvoli et al., 1994) as well as from other plant species. The present work shows that the high CO_2 pretreatment that significantly reduced natural fungal decay in table grapes stored at 0 °C prevented the large increase in *VcPAL* transcript levels observed in non-treated grapes (Fig. 3A). The *PAL* gene expression is also elicited in this way in grapevine leaves infected with *B. cinerea* (Bezier et al., 2002). Furthermore, the increase in PAL activity was higher in non-treated grapes (Fig. 3B). PAL was one of the first plant "defense genes" identified, and both PAL mRNA levels and PAL activity could be induced by a wide array of environmental cues, such as pathogen attack, low temperatures, UV irradiation, mechanical wounding, and light (Lawton and Lamb, 1987; Gläβgen et al., 1998; Kamo et al., 2000; Sanchez-Ballesta et al., 2000). PAL expression and activity may therefore be considered a good marker to investigate the process whereby high CO_2 levels effectively control the grape decay. The PAL activation observed in untreated cherimoya fruit after prolonged storage at chilling temperature was reduced by an effective high CO_2 pretreatment (Maldonado et al., 2002). Likewise, in mandarin fruit an effective heat treatment prevented the increase in PAL activity that normally occurs around the necrotic zones of fruits when stored at low temperatures (Martínez-Téllez and Lafuente, 1997; Sanchez-Ballesta et al., 2000). The results of the present work, then, reinforce the idea that PAL may be implicated in defense responses against natural fungal infection in non-treated grapes but not in the mechanisms operating in treated grapes to reduce total decay.

In grapevine, the copy number of CHS has been estimated at three to four. The predicted partial VcCHS amino-acid sequence shared more than 90% identity with the three different grapevine CHS deposited in the databases, showing the closest homology to CHS2 (Goto-Yamamoto et al., 2002). The 3-day CO_2 pretreatment also reduced the increase in the *VcCHS* transcript levels observed in non-treated grapes (Fig. 3A). When it was compared with *VcPAL* gene expression in non-treated grapes stored at 0 °C, the increase in *VcCHS* mRNA was later and progressive, but the maximum in both *PAL* and *CHS* gene expression was observed by day 28. The activities of CHS are largely regulated at the transcription level. The expression of CHS genes is controlled by developmental stages of the plant, as well as by biological and environmental stresses such as low temperature, pathogen attack and wounding (Junghans et al., 1993; Leyva et al., 1995; Seki et al., 1999). Table grapes stored at low temperatures in air respond to natural fungal decay with a large increase in *PAL* and *CHS* gene expression, PAL activity and total anthocyanin content. In this respect, the lower *VcPAL* and *VcCHS* mRNA levels in CO_2-treated fruit might constitute cellular signals indicating a lower rate of stress imposition. Furthermore, this protective effect of high CO_2 pretreatment is concomitant with a decrease in the induction of *APX* gene expression. We have also isolated a partial cDNA encoding a cytosolic *APX*. At present, there are many reports about cytosolic APX in different plants and fruits (Mittler and Zilinskas, 1991; Ishikawa et al., 1995; Kim and Chung, 1998), but there are no reports on table grapes related to *APX* gene. In the present study, the analysis of *APX* gene expression revealed that transcript levels increased slightly both in treated and non-treated grapes, but a sharp increase was observed after 28 days in non-treated grapes where total decay was evident (Romero et al., 2006). These results indicate differences in the mechanisms operating in treated and non-treated grapes, suggesting that APX participate in removing the putative high levels of H_2O_2 in non-treated grapes. APX seems to be activated during compatible interactions (Garcia-Limones et al., 2002), and many reports indicated that it is even inhibited during resistance or during pathogen-induced programmed cell death (Mittler et al., 1998).

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

The evidences provided here suggest that the effectiveness of high CO_2 pretreatment to control fungal decay maintaining fruit quality after prolonged low-temperature storage is the result of ability to prevent the formation of ROS rather than their inactivation once formed. Further analysis is needed to ascertain the role of anthocyanin induced during low-temperature storage and to identify responses associated with the antioxidant systems that participate in the beneficial effect of CO_2 treatment.

Acknowledgements

This work was supported by research grants (AGL2005-04502/ALI and AGL2002-02308) from the CICYT (Spain). I.R. and MT.S.-B. were supported by a postgraduate and postdoctoral I3P contract from the CSIC, respectively. The authors are grateful to Frutaria SAT for providing tables grapes and to Agrotecnica for industrial assistant.

References

Alscher RG, Donahue JL, Cramer CL. Reactive oxygen species and antioxidants: relationships in green cells. Physiol Plant 1997;100:224–33.

Artés-Hernández F, Tomás-Barberán FA, Artés F. Modified atmosphere packaging preserves quality of SO_2-free 'Superior seedless' table grapes. Postharvest Biol Technol 2006;39:146–54.

Bezier A, Lambert B, Baillieul F. Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. Eur J Plant Phatol 2002;108:111–20.

Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

Crisosto CH, Garner D, Crisosto G. Carbon dioxide-enriched atmospheres during cold storage limit losses from *Botrytis* but accelerate rachis browning of 'Redglobe' table grapes. Postharvest Biol Technol 2002;26:181–9.

Dixon RA, Pavia NL. Stress-induced phenylpropanoid metabolism. Plant Cell 1995;7:1085–97.

Faragher JD, Chalmers DJ. Regulation of anthocyanin synthesis in apple skin. 3. Involvement of phenylalanine ammonia-lyase. Aust J Plant Physiol 1977;4:133–41.

Garcia-Limones C, Hervas A, Navas-Cortés J, Jiménez-Diaz RM, Tena M. Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum f*. sp *Ciceris*. Physiol Mol Plant Pathol 2002;61:325–37.

Gil MI, Holcroft DM, Kader AA. Changes in strawberry anthocyanins and other polyphenols in response to carbon dioxide treatments. J Agric Food Chem 1997;45:1662–7.

Gil MI, Ferreres F, Tomas-Barberan FA. Effect of post-harvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. J Agric Food Chem 1999;47:2213–7.

Gläβgen WE, Rose A, Madlung J, Koch W, Gleitz J, Seitz HU. Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. Planta 1998;204:490–8.

Goto-Yamamoto N, Wan GH, Masaki K, Kobayashi S. Structure and transcription of three chalcone synthase genes of grapevine (*Vitis vinifera*). Plant Sci 2002;162:867–72.

Govrin EM, Levine A. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biol 2000;10:751–7.

Holton TA, Cornish EC. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 1995;7:1071–83.

Ishikawa T, Sakai K, Takeda T, Shigeoka S. Cloning and expression of cDNA encoding a new type of ascorbate peroxidase from spinach. FEBS Lett 1995;367:28–32.

Junghans H, Dalkin K, Dixon RA. Stress responses in alfalfa (*Medicago sativa* L.). 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol Biol 1993;22:239–53.

Kalt W, Forney CF, Martin A, Prior RL. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. J Agric Food Chem 1999;47:4638–44.

Kamo T, Hirai N, Tsuda M, Fujioka D, Ohigashi H. Changes in the content and biosynthesis of phytoalexins in banana fruit. Biosci Biotechnol Biochem 2000;64:2089–98.

Kawakami S, Matsumoto Y, Matsunaga A, Mayama S, Mizuno M. Molecular cloning of ascorbate peroxidase in potato tubers and its response during storage at low temperature. Plant Sci 2002;163:829–36.

Kim IJ, Chung WI. Molecular characterization of a cytosolic ascorbate peroxidase in strawberry fruit. Plant Sci 1998;133:69–77.

Lawton MA, Lamb CJ. Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol Cell Biol 1987;7:335–41.

Leyva A, Jarillo JA, Salinas J, Martinez-Zapater JM. Low-temperature induces the accumulation of *phenylalanine ammonia-lyase* and *chalcone synthase* mRNAs of *Arabidopsis thaliana* in a light-dependent manner. Plant Physiol 1995;108:39–46.

Li J, Ou-Lee T, Raba R, Amundson RG, Last R. *Arabidopsis* flavonoid mutant are hypersensitive to UV-B irradiation. Plant Cell 1993;5:171–9.

Maldonado R, Molina-García AD, Sanchez-Ballesta MT, Escribano MI, Merodio C. High CO_2 atmosphere

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

modulating the phenolic response associated with cell adhesion and hardening of *Annona cherimola* fruit stored at chilling temperature. J Agric Food Chem 2002;50:7564–9.

Mancinelli AL. The photoregulation of anthocyanin synthesis. In: Shropshire Jr V, Mohr H, editors. Encyclopedia of plant physiology. Springer; 1983. p. 640–61.

Martínez-Téllez MA, Lafuente MT. Effect of high-temperature conditioning on ethylene, phenylalanine ammonialyase, peroxidase and polyphenol oxidase activities in flavedo of Fortune mandarin fruit. J Plant Physiol 1997;150:674–8.

Mehdy MC, Sharma YK, Sathasivan K, Bays NW. The role of activated oxygen species in plant disease resistance. Plant Physiol 1996;98:365–74.

Mittler R, Zilinskas BA. Molecular cloning and nucleotide sequence analysis of a cDNA encoding pea cytosolic ascorbate peroxidase. FEBS Lett 1991;289:257–9.

Mittler R, Feng X, Cohen M. Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen-induced programmed cell death in tobacco. Plant Cell 1998;10:461–71.

Mori K, Sugaya S, Gemma H. Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. Sci Horticul 2005;105:319–30.

Remon S, Ferrer A, López-Buesa P, Oria R. Atmosphere composition effects on Burlat cherry colour during cold storage. J Sci Food Agric 2004;84:140–6.

Retamales J, Defilippi BG, Arias M, Castillo P, Manríquez D. High-CO_2 controlled atmospheres reduce decay incidence in Thompson Seedless and Red Globe table grapes. Postharvest Biol Technol 2003;29:177–82.

Rice-Evans CA, Miller NJ, Paganda G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol Med 1996;20:933–56.

Romero I, Sanchez-Ballesta MT, Maldonado R, Escribano MI, Merodio C. Expression of class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO_2 pretreatment. Postharvest Biol Technol 2006;41:9–15.

Salzman RA, Fujita T, Zhu-Salzman K, Hasegawa PM, Bressan RA. An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol Biol Rep 1999;17:11–7.

Sanchez-Ballesta MT, Lafuente MT, Zacarias L, Granell A. Involvement of phenylalanine ammonia-lyase in the response of Fortune mandarin fruits to cold temperature. Physiol Plant 2000;108:382–9.

Sanchez-Ballesta MT, Jiménez JB, Romero I, Orea JM, Maldonado R, González-Ureña A, et al. Effect of high CO_2 pretreatment on quality, fungal decay and molecular regulation of stilbene phytoalexin biosynthesis in stored table grape. Postharvest Biol Technol 2006;42:209–16.

Seki H, Nagasugi Y, Ichinose Y, Shiraishi T, Yamada T. Changes in *in vivo* DNA–protein interactions in pea phenylalanine ammonia-lyase and chalcone synthase gene promoter induced by fungal signal molecules. Plant Cell Physiol 1999;40:88–95.

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol Biol 1994;24:743–55.

Tanaka Y, Kojima M, Uritani I. Properties, development and cellular-localization of cinnamic acid 4-hydroxylase in cutinjured sweet potato. Plant Cell Physiol 1974;15:843–54.

Tierens KF, Thomma BP, Bari RP, Garmier M, Eggermont K, Brouwer M, et al. Esa1, an *Arabidopsis* mutant with enhanced susceptibility to a range of necrotrophic fungal pathogens, shows a distorted induction of defense responses by reactive oxygen generating compounds. Plant J 2002;29:131–40.

van der Sluis AA, Dekker M, de Jager A, Jongen WMF. Activity and concentration of polyphenolic antioxidants in apple: Effect of cultivar, harvest year, and storage conditions. J Agric Food Chem 2001;49:3606–13.

Veazie PP, Collins JK. Quality of erect-type blackberry fruit after short intervals of controlled atmosphere storage. Postharvest Biol Technol 2002;25:235–9.

Winkel-Shirley B. Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 2001;126:485–93.

Wrolstad RE. Colon and pigment analysis in fruit products (Bulletin 624). Corvallis, Oregon: Oregon Agricultural Experimental Station; 1976.

Yahia EM, Nelson KE, Kader AA. Postharvest quality and storage life of grapes as influenced by adding carbon monoxide to air or controlled atmospheres. J Am Soc Hortic Sci 1983;108:1067–71.

Yoshimura K, Yabuta Y, Ishikawa T, Shigeoka S. Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stress. Plant Physiol 2000;123:223–33.

Please cite this article as: Romero I, et al. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO_2-treated table grapes stored at low temperature. J Plant Physiol (2007), doi:10.1016/j.jplph.2006.12.011

ARTÍCULO 4

Individual anthocyanins and their contribution to total antioxidant capacity in response to low temperature storage and high CO_2 in Cardinal table grapes.

Irene Romero; María T Sanchez-Ballesta; María I Escribano; Carmen Merodio

Postharvest Biology and Technology **(aceptado)**

RESUMEN

En este estudio, hemos analizado los perfiles de los antocianos individuales en la piel de la uva de mesa y su contribución a la capacidad antioxidante total (TAC) en respuesta a bajas temperaturas (0ºC) y altos niveles de CO_2 (20 % durante 3 días). También se determinó el análisis de los parámetros representativos de color de esta variedad roja-violeta. La composición de antocianos fue determinada usando HPLC-DAD-MS. La contribución de los antocianos individuales al valor TAC de uvas de mesa fue calculado basándonos en su concentración y capacidad antioxidante medido como valor TEAC (pendiente de antocianos/ pendiente Trolox). Los análisis cromatográficos identificaron seis antocianos en la piel de uvas de mesa Cardinal, incluyendo pelargonidina-3-glucósido (Pg-3-G). Tiempos cortos de almacenamiento a 0ºC en aire tuvieron como efecto incrementar la concentración de cada antociano identificado. Después de 3 días a 0ºC, las uvas no tratadas presentaron el mayor contenido en antocianos (27.55 mg/100gFW) y presentaron el mayor valor TAC (52.45 mM/100gFW). La peonidina-3-glucósido (Pn-3-G), antociano predominante en esta variedad de uva roja, fue la principal responsable del aumento de los valores TAC en uvas de mesa no tratadas. La Pn-3-G tuvo un bajo valor de TEAC (1.73 mM), pero su contribución podría ser explicada por el gran aumento encontrado en el contenido de este antociano los primeros días de almacenamiento a 0ºC. Por el contrario, al final del tratamiento con CO_2, el contenido de Pn-3-G no cambió, y tanto el contenido de antocianos como el valor TAC de uvas de mesa tratadas con CO_2 fueron similares a los encontrados en uvas antes de la conservación. Además, el tratamiento con CO_2 llevó a un aumento significativo en el contenido de Pg-3-G, concomitante con una bajada pronunciada en delfinidina-3-glucósido (Dp-3-G) y menores

descensos en petunidina-3-glucósido (Pt-3-G) y malvidina 3-glucósido (Mv-3-G). Estos efectos de los altos niveles de CO_2 en el perfil de antocianos individuales se fueron perdiendo progresivamente cuando las uvas fueron transferidas a aire. De hecho, después del almacenamiento prolongado a bajas temperaturas, se cuantificaron valores de TAC similares en uvas no tratadas y aquellas expuestas al pretratamiento con 20% de CO_2. Estos datos abren nuevas perspectivas sobre el efecto de bajas temperaturas y altos niveles de CO_2 en la concentración de antocianos individuales identificados en uvas de mesa Cardinal.

Available online at www.sciencedirect.com
ScienceDirect
Postharvest Biology and Technology xxx (2008) xxx–xxx
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes

Irene Romero, María T. Sanchez-Ballesta, María I. Escribano, Carmen Merodio *

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frío, IF-CSIC, José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain

Received 20 September 2007; accepted 30 November 2007

Abstract

In this study, we have analyzed the profiles of individual anthocyanins in the skin of cardinal table grapes and their contribution to the total antioxidant capacity (TAC) in response to low temperature (0 °C) and high CO_2 levels (20% for 3 days). An analysis of the representative colour parameters of this red-violet variety was also determined. The anthocyanin composition was determined using high-performance liquid chromatography coupled to diode array detector and mass spectrometry (HPLC–DAD–MS). The contribution of individual anthocyanins to the TAC value of table grapes was calculated on the basis of their concentration and antioxidant capacity measured as the TEAC value (slope of the anthocyanin/slope of Trolox). Chromatographic analysis identified six anthocyanins, including pelargonidin 3-glucoside (Pg-3-G), in the skin of cardinal table grapes. Short-term storage at 0 °C in air had an increasing effect on the concentration of each of the identified anthocyanins. After 3 days at 0 °C, untreated grapes had the highest anthocyanin content (27.55 mg/100 g FW) and displayed the largest TAC value (52.45 mM TE/199 g FW). Peonidin 3-glucoside (Pn-3-G) was the predominant anthocyanin, and it was mainly responsible for the rise in the calculated TAC value in untreated grapes. Pn-3 G had a low average TEAC value (1.73 mM), but its contribution could be explained by the sharp increase in the content of this anthocyanin the first days of storage at 0 °C. In contrast, the Pn-3-G content in grapes at the end of the 3-day CO_2 treatment did not change, and both the total anthocyanin content and the calculated TAC value remained significantly constant in comparison to the levels in pre-stored grapes. In addition CO_2 treatment had a positive effect on the amount of Pg-3-G concomitant with a pronounced decline in delphinidin 3-glucoside (Dp-3-G) and smaller decreases in petunidin 3-glucoside (Pt-3-G) and malvidin 3-glucoside (Mv-3-G). These effects of high CO_2 levels on the profile of individual anthocyanins were progressive lost when grapes were transferred to air. Indeed, after prolonged low-temperature storage when the colour of the berries become dark violet, similar calculated TAC values were quantified in untreated grapes and in those exposed to 20% CO_2. These data provide new insights about the effect of low temperature and high CO_2 levels on the concentration of the individual anthocyanins identified in cardinal table grapes.

Keywords: Table grapes; Post-harvest technology; Carbon dioxide; Low temperature; Anthocyanins profile; Total antioxidant capacity

1. Introduction

Low temperature is one of the most effective technologies to extend the post-harvest storage life of table grapes. Although *Vitis vinifera* is tolerant to chilling, activation of the defense in responses to stress have been reported in cardinal table grapes. Specifically, we observed a transitory increase in the abundance of *PAL* and *CHS* transcripts during the first 3 days of storage at 0 °C in the skin of untreated grapes, which was accompanied by an increase in total anthocyanin content (Sanchez-Ballesta et al., 2007). Indeed, cold temperature has been seen to increase anthocyanin levels in several growing plant species (Christie et al., 1994; Faragher, 1983; Oren-Shamir and Levi-Nissim, 1997; Shichijo et al., 1993; Stiles et al., 2007). Although this phenomenon appears to be relatively common, there is still some uncertainty concerning the significance of increased anthocyanin production during post-harvest storage at low temperature. Indeed, the effect of low temperature on the concentration and distribution of anthocyanin pigments, and on their antioxidant activity, has yet to be fully characterized.

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: merodio@if.csic.es (C. Merodio).

0925-5214/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.postharvbio.2007.11.009

Anthocyanins are usually present in plants as a mixture of major and minor compounds. Their separation and identification requires the use of reversed-phase high-performance liquid chromatography (HPLC) with diode array detection (DAD), which is effective in analyzing these pigments (Hong and Wrolstad, 1990). However, the natural variability of different anthocyanins is large and some of them present similar retention times and spectral characteristics, making their identification by HPLC–DAD alone difficult. Therefore other techniques are often needed to accurately identify and characterize minor pigments. Among these techniques, mass spectrometry and MS^n analysis, coupled to the HPLC–DAD, are widely used to identify anthocyanins in different biological tissues (De Pascual-Teresa et al., 2002).

Several assays have been introduced to measure total antioxidant activity in food extracts and as such, it is often difficult to compare the results obtained for the same product in different studies. Furthermore, since antioxidant activity is derived from the delicate balance between antioxidants and prooxidants components, the total antioxidant capacity (TAC) of biological tissues in response to environmental conditions is sometimes not uniform. Hence, to calculate the TAC of table grapes during post-harvest storage we have considered the sum of the antioxidant capacity of the individual anthocyanins determined in the sample. The antioxidant activity of many anthocyanins was previously reported in terms of their Trolox equivalent antioxidant capacity (TEAC) (Awika et al., 2004; Solomon et al., 2006). However, differences in the protocols and calculation methods (De Beer et al., 2006).produce different TEAC values for the respective anthocyanins. Furthermore, while these values are useful to determine the relative importance of the respective phenolic compounds, published data cannot be used to calculate the contribution of individual compounds to the TAC of a given sample. Hence, the TEAC values of pure anthocyanin reference standards have been measured in this work using the same protocol as that employed to determine the TAC of table grapes stored under different environmental conditions.

Anthocyanins are also responsible for many red, violet and blue colours in fruits and flowers (Harbone and Grayer, 1988; Remon et al., 2004). Pelargonidins are primarily responsible for the orange, salmon, pink and red colour of the fruit, while the magenta and crimson comes from the cyanidins, and the purple, mauve, and blue are due to the delphinidins present. Since colour is an important parameter in berry quality, the association between colour and anthocyanin pigments profiles has been evaluated during post-harvest storage of table grapes. In this work, we applied the CIELAB colorimetric system to assess the changes in skin colour of untreated and CO_2-treated grapes during low-temperature storage.

The aim of the present work was to analyze the individual anthocyanins in red-violet table grapes and to define their changes in response to low-temperature storage (0 °C) after harvest. We also assessed how table grapes respond to high CO_2 levels (3 days, 20%) at 0 °C, both at the end of the treatment and when grapes were transferred to air. To associate anthocyanin production with important metabolic aspects of the fruit, we analyzed the contribution of individual anthocyanins to the TAC, as well as their implication in the colour of the skin of the grapes when exposed to CO_2 or not. This information is essential to evaluate the efficiency of post-harvest technologies whose goals should be to maintain the health and quality of the intact fruit.

2. Materials and methods

2.1. Plant material

Early-harvesting mature table grapes (*V. vinifera* L. cv. Cardinal) from Sevilla (Spain) were used (12.7% total soluble solids, 0.81% tartaric acid). Forced-air pre-cooled bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 °C and 95% relative humidity (RH) in two sealed neoprene containers of 1 m^3 capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container. One lot was stored in air for 33 days (untreated fruit) and the other under a gas mixture of 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. The CO_2 concentration was maintained throughout the pretreatment and was measured daily using an automated gas chromatography system equipped with a thermal conductivity detector and a Poraplot Q column (Varian Chrompack CP20033P). After 3 days, the CO_2-treated grapes were transferred to air under the same conditions as the untreated fruit until the end of the storage period. Ten clusters were sampled periodically. The berries from five clusters (approximately 300 g each cluster) were peeled and the skin was mixed, frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C for further analysis. For colour analysis, 45 berries were used that were randomly removed from the other five clusters and distributed into three replicates of 15 berries each.

2.2. Extraction of anthocyanins

Frozen skin samples (0.5 g) were homogenized with 0.5 mL methanol containing 0.01% HCl by ultra-sonication for 10 min. The extract was centrifuged at 4000 × *g* and the supernatant was removed. This step was repeated twice with the same solvent system until no more pigment was extracted and the solvent remained clear. The combined supernatants were filtered through a 0.45 μm nylon membrane filter (Millipore), to remove the solids residues, and the filtered material was evaporated to dryness with N_2 gas before resuspending in an equal volume of acidified water containing 4.5% formic acid (v/v) and acetonitrile (95:5, v/v) for later HPLC–DAD–MS analysis.

2.3. HPLC–DAD–MS analyses

Aliquots of extracted anthocyanins (50 μL) were analyzed using a liquid chromatography/mass selective detector (LC/MSD) system coupled in series to a photodiode array detector (DAD, G1315B) consisting of a quaternary pump (G1311A), a vacuum degasser, a well-plate autosampler (G1313A) and a thermostat controlled column compartment controlled by software LC/MS ChemStation Revision A.08.03 from Agilent Technologies 1100 series (Waldbronn, Germany). Samples

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009

were injected at room temperature (20 °C) and the components were separated using a reverse-phase C_{18} column (150 mm × 4.6 mm, 5 μm particle size, Scharlab, Barcelona, Spain). The mobile phase consisted of acidified water containing 4.5% formic acid (v/v, A) and acetonitrile (B). The gradient used commenced with 5% phase B at time 0, 5–8% at 5–10 min, 8–10% at 10–15 min, 10–15% at 15–22 min, 15–25% at 22–27 min, 25–40% at 27–30 min, 40–73% at 30–33 min, 73–95% at 33–34 min, 95–5% at 34–41 min. The flow rate was 0.9 mL/min, and the wavelength of detection was set at 520 nm. Scanning was also performed from 450 to 800 nm in 2 nm steps. Electrospray mass spectrometric analyses were carried out in a positive ion mode using a quadrupole mass spectrometer (G1946D) fitted with an atmospheric pressure electrospray ionization source (API-ES) with a fragmentation voltage of 140 V. The conditions of the spray chamber were nebulizer, 45 psig; dry gas (N_2), 12 L/min; gas temperature, 300 °C; capillary voltage (positive), 3000 V. Spectra were recorded between *m/z* 287 and 494. The anthocyanin peaks were identified by comparison with molecular and main fragment ion values previously described in the literature (Muñoz-Espada et al., 2004; De Beer et al., 2006) and confirmed by comparison of commercially available anthocyanins standards. The anthocyanin concentrations were determined by the peak areas of the respective ion chromatograms extracted for delphinidin 3-*O*-glucoside (Dp-3-G), cyanidin 3-*O*-glucoside (Cy-3-g), petunidin 3-*O*-glucoside (Pt-3-G), pelargonidin 3-*O*-glucoside (Pg-3-G), peonidin 3-*O*-glucoside (Pn-3-G) and malvidin 3-*O*-glucoside (Mv-3-G), and using a standard curve derived from the commercial anthocyanins (Extrasynthese, France). The range of the calibration curves, with at lest five points for each standard anthocyanin, took into account the relative abundance of table grape anthocynins: 0–2 μg/mL for Pg-3-G, 1–10 μg/mL for Cy-3-G, 2–20 μg/mL for Dp-3-G and Mv-3-G and 2–300 μg/mL for Pn-3-G. The quantification for Pt-3-G was carried out in Pg-3-G equivalents and individual anthocyanin levels were expressed as mg/100 g fresh weight (FW).

2.4. Colour

Berry skin colour was measured at three different positions around the equator of the grape using the Hunter Lab System and a Minolta CR200™ colorimeter (Minolta Camera Co., Osaka, Japan). Results were given in Commission Internationale de l'Eclairage *L** (lightness), *a**, and *b** (CIELAB) colour space coordinates. The chroma $(a^2 + b^2)^{1/2}$ and hue angle $(\tan^{-1} b/a)$ were calculated.

2.5. Radical cation ABTS scavenging capacity

Trolox (6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid, Hoffman-La Roche) and standard anthocyanins, Dp-3-G, Cy-3-G, Pn-3-G, Pg-3-G and Mv-3-G (Extrasynthese, France) were diluted with ethanol at different concentrations to evaluate their antioxidant capacity, in a linear range, by the radical cation 2,2′-azinobis(3-ethylbenzothiazolone 6-sulphonate) ($ABTS^{\bullet+}$) as described by Re et al. (1999). The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0–70 (±0.02) at 734 nm and equilibrated at 30 °C. After adding 990 μL of diluted $ABTS^{\bullet+}$ solution to 10 μL aliquots of different concentrations of Trolox and standard anthocyanins (μM), the absorbance reading was taken at 30 °C exactly 1 min after the initial mixing, and then for up to 6 min. Appropriate solvent blanks were run in each assay. The inhibition of absorbance at 734 nm is calculated and plotted as a function of the antioxidant concentration, and of the inhibition produced by Trolox as the standard reference compound.

The TAC contribution (mM TE/100 g FW) of individual anthocyanins in the skin of table grape was calculated from their anthocyanins content (mg/100 g FW) and TEAC values (mM) as described by De Beer et al. (2006):

- TEAC = slope (test compound)/slope (Trolox).
- TAC contribution = [compound] × TEAC.

2.6. Statistical analyses

Data from at least three replicates per sampling period were subjected to an analysis of variance (ANOVA, Statgraphics program, STSC, Rockville, MD), and a multiple variance analysis was used to determine the significance of the data at $P \leq 0.05$. Two-way analysis of variance was performed using the LSD test procedure with type III sums of squares and a confidence level of 95% (Statgraphics program, STSC, Rockville, MD). The main effects of high CO_2 treatment, time of storage at 0 °C, and the CO_2 treatment × time interaction on fruit were assessed.

3. Results

3.1. Identification of anthocyanins

By combining HPLC–DAD and the analysis of the MS spectra, six anthocyanins were identified in Cardinal table grapes (Fig. 1), whose retention times, molecular ions and main fragment ions are summarized in Table 1. Of these, peak 5 was associated with a strong signal in the MS detector and it corresponded to Pn-3-G. The MS spectrum revealed a molecular ion at *m/z* 433.3 associated with peak 4 and the main ion fragment produced was 271, corresponding to Pg-3-G. Additionally, a comparison of the molecular ions and ion products of these anthocyanins with those of commercially available anthocyanin

Table 1
Chromatographic and spectrophotometric characteristics of each identified anthocyanins in cardinal table grape

Peak number	Retention time (min)	Molecular ion (*m/z*)	Main fragment ions	Peak assignment
1	4.78 ± 0.16	465.3	303	Dp-3-G
2	7.14 ± 0.32	449.2	287	Cy-3-G
3	8.95 ± 0.39	479.4	317	Pt-3-G
4	10.35 ± 0.40	433.3	271	Pg-3-G
5	12.12 ± 0.39	463.3	301	Pn-3-G
6	14.17 ± 0.35	493.4	331	Mv-3-G

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009

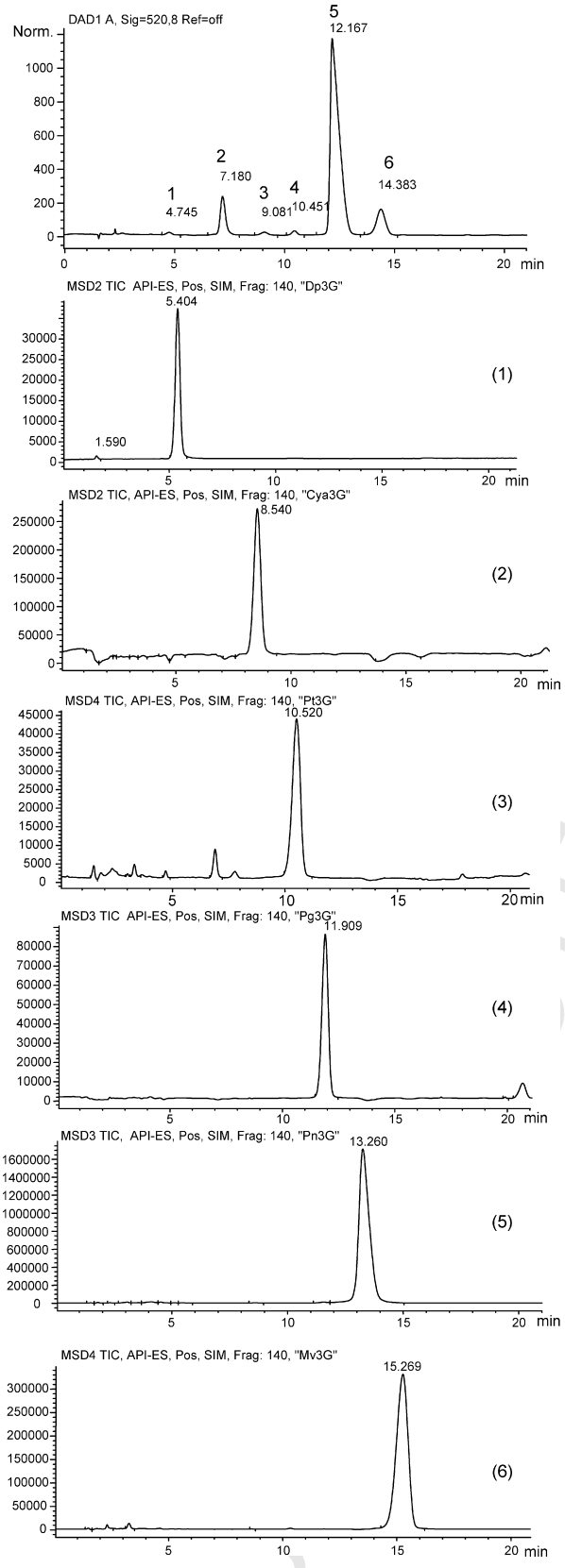


Fig. 1. HPLC–DAD chromatogram (520 nm) and the ion chromatograms extracted at *m/z* corresponding to the molecular ion of each identified anthocyanins in cardinal table grape: (1) delphinidin-3-glucoside; (2) cyanidin-3-glucoside; (3) petunidin-3-glucoside; (4) pelargonidin-3-glucoside; (5) peonidin-3-glucoside; (6) malvidin-3-glucoside.

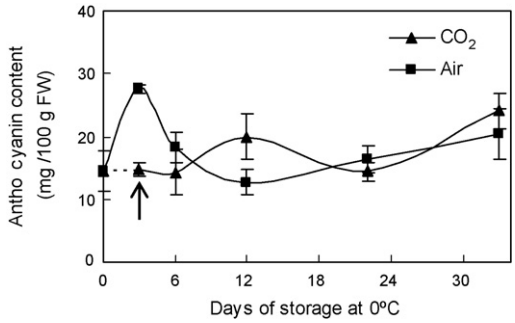


Fig. 2. Changes in anthocyanin content as result of the sum of the individual anthocyanins identified and quantified in untreated and CO_2-treated cardinal table grapes during storage at 0 °C. The dotted line indicates the short period of high CO_2 treatment. The row indicates the transfer of CO_2-treated fruit to air. The results are the mean of three replicate samples ± S.E.

standards confirmed the identification of Dp-3-G, Cy-3-G, Pn-3-G, Pg-3-G, Mv-3-G and Pt-3-G.

3.2. Content and distribution of individual anthocyanins in table grapes in response to low temperature and short-term exposure to high levels of CO_2

The change in the anthocyanin content as result of the sum of the six individual anthocyanins during storage at 0 °C was examined (Fig. 2), and the anthocyanin levels in CO_2 treated grapes were quantified at the end of the treatment and when grapes were transferred to air. While 3 days of storage in air induced a sharp increase in the anthocyanin content from 14.55 to 27.55 mg/100 g FW, exposure to high levels of CO_2 did not cause any change with respect to the values of pre-stored grapes. After 6 days storage at 0 °C, the elevated anthocyanin content observed in untreated grapes decreased, reaching values similar to those found in CO_2-treated ones. In contrast, the anthocyanin content of CO_2-treated grapes transferred to air increased after 12 days and was still higher than that in untreated grapes at the end of the storage period.

The individual concentration of anthocyanins found in table grapes at the end of the CO_2 treatment and in those stored in air was determined at the same chronological age (3 days) (Table 2). Pn-3-G was the predominant anthocyanin found in this variety, with values of 12.78 mg/100 g FW in pre-stored table grapes. In untreated fruit stored in air for 3 days, there was an important increase in Pn-3-G content (+97%), reaching values of 25.20 mg/100 g FW. There was also a sharp increase in the minor anthocyanin Pg-3-G (+141%), from 0.061 to 0.147 mg/100 g FW. The first days of storage at 0 °C had a less pronounced effect on Cy-3-G (+49%), Dp-3-G (+48%) and Pt-3-G (+25%), and only a moderate effect on the Mv-3-G content (+18%). While exposure for 3 days to high CO_2 treatment did not effect the Pn-3-G content (0%) and it had only a moderate effect on the Cy-3-G content (+24%), it sharply increased the Pg-3-G (+200%). In contrast to the increase observed in grapes stored in air, high CO_2 treatment caused a pronounced decrease in Dp-3-G (−64%) and smaller decreases in Mv-3-G (−21%) and Pt-3-G (−27%) with respect to the content in pre-stored grapes.

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009

Table 2
Individual anthocyanin content (mg/100 g FW) in untreated and CO_2-treated grapes after 3 days at 0 °C

	Days at 0 °C				
	0 day	3-day air	Δ (%)[a]	3-day CO_2	Δ (%)[b]
Pn-3-G	12.78 ± 3.09 a[c]	25.20 ± 0.33 b	+97	13.08 ± 0.84 a	0
Cy-3-G	0.41 ± 0.01 a	0.61 ± 0.12 b	+49	0.51 ± 0.06 a,b	+24
Pg-3-G	0.061 ± 0.003 a	0.147 ± 0.007 b	+141	0.183 ± 0.009 c	+200
Mv-3-G	1.14 ± 0.10 a,b	1.35 ± 0.14 b	+18	0.90 ± 0.18 a	−21
Dp-3-G	0.123 ± 0.015 b	0.182 ± 0.004 c	+48	0.044 ± 0.015 a	−64
Pt-3-G	0.048 ± 0.002 b	0.060 ± 0.005 c	+25	0.035 ± 0.002 a	−27

[a] Delta between those two time points $t = 0$ and $t =$ 3-day air.
[b] Delta between those two time points $t = 0$ and $t =$ 3-day CO_2.
[c] Different letter in a row indicate a significant difference ($P \leq 0.05$).

The change in the proportion of individual anthocyanins relative to the total anthocyanin content was determined in untreated grapes during storage at 0 °C and in CO_2 treated grapes at the end of the treatment and when the grapes were transferred to air (Fig. 3). Pn-3-G was the main anthocyanin, accounting for 87.8% of anthocyanin content in pre-stored grapes. After 3 days storage at 0 °C, a rise in the percentage of Pn-3-G was observed in untreated grapes. By contrast, Pn-3-G content was similar to the values found in pre-stored grapes after the CO_2-treatment. Interestingly, at the end of the 3-day CO_2 treatment there was a pronounced decrease in the anthocyanins synthesized directly from dihydromyricetin in the grapes, namely Dp-3-G, Mv-3-G and Pt-3-G, concomitant with a marked increase in the 3 *O*-glucoside of pelargonidin. After 6 days storage at 0 °C, the sharp rise both in the anthocyanin content and in the percentage of Pn-3-G observed in the first days of storage in untreated grapes decreased, reaching levels similar to those observed in the CO_2-treated fruit. Thus, our results showed that when CO_2-treated grapes were transferred to air, the percentage of Cy-3-G and Pg-3-G dropped sharply while the proportion of Mv-3-G, Dp-3-G and Pt-3-G began to increase, reaching similar values to those detected in untreated grapes after 12 days in storage. Thereafter, the trend in the percentage of individual anthocyanins was virtually identical in untreated grapes and those exposed to CO_2, finally reaching the same proportions.

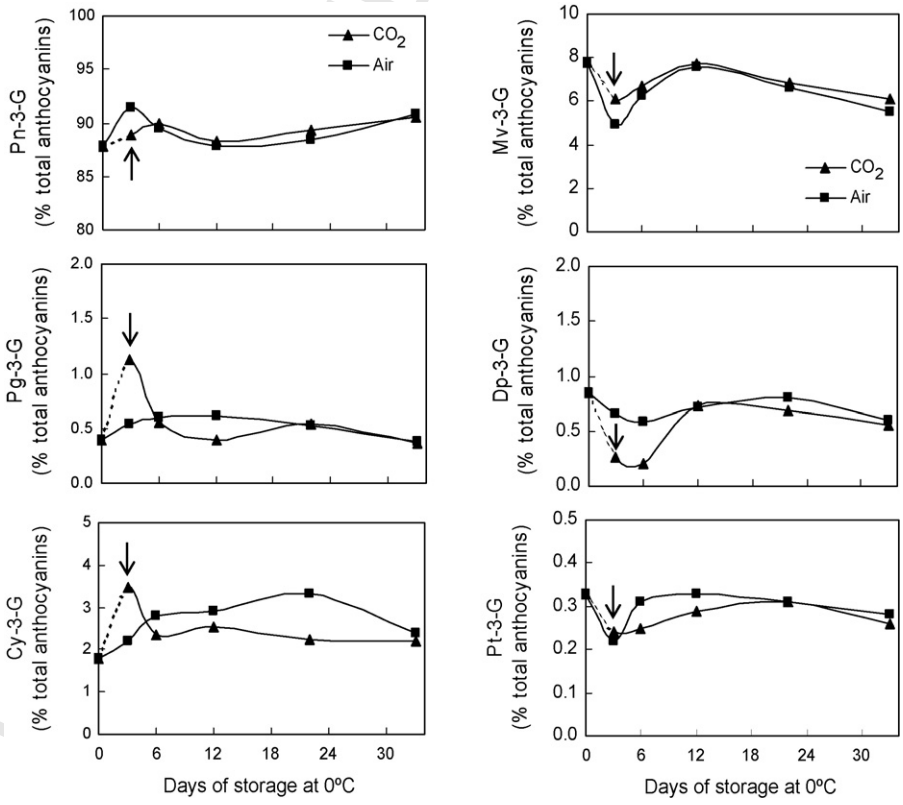


Fig. 3. Percentage of individual anthocyanins relative to the total anthocyanin content in untreated and CO_2-treated cardinal table grapes during storage at 0 °C. The dotted line indicates the short period of high CO_2 treatment and the row indicates the transfer of CO_2-treated fruit to air.

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009

3.3. Skin colour changes in untreated grapes and those exposed to CO_2 during storage at 0 °C

We determined the evolution of the parameters L^*, a^*, b^*, chroma and the hue angle in untreated and CO_2-treated grapes during storage at 0 °C (Fig. 4). The skin of untreated and CO_2-treated berries showed a similar evolution in their red-violet colour during low-temperature storage. The values of parameter a^* and chroma showed a progressive decrease in the first days of storage at 0 °C, and were higher in untreated than in CO_2-treated grapes. This decrease was particularly notable after 22 days when they reached their lowest values. On the contrary, the values of the parameter b^* and the hue angle undergo an opposite effect over the storage period and they were higher in CO_2-treated than in untreated grapes. After 22 days of storage at 0 °C, the values of the parameter b^* and hue angle were very near 0, and the L^* parameter reached minimum values, indicating that at this time the colour of untreated and CO_2-treated berries was dark violet.

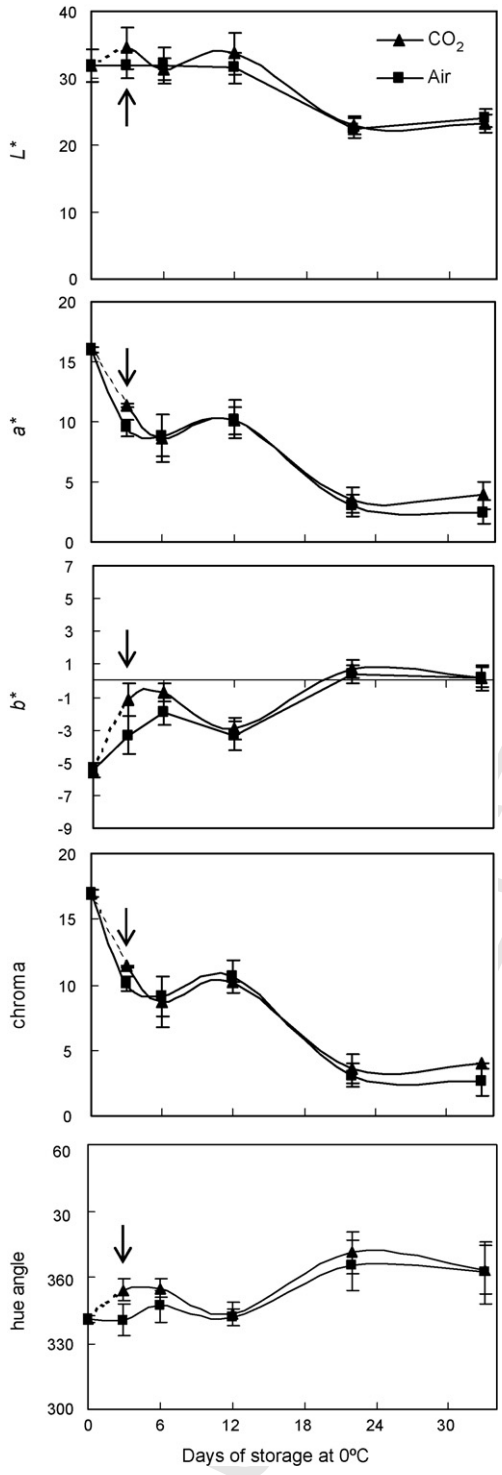


Fig. 4. Evolution of the L^*, a^*, b^*, chroma and hue angle in untreated and CO_2-treated cardinal table grapes during storage at 0 °C. The dotted line indicates the short period of high CO_2 treatment and the row indicates the transfer of CO_2-treated fruit to air. The results are the mean of three replicate samples ± S.E.

3.4. The antioxidant capacity of table grapes in response to low temperature and short-term exposure to high CO_2

The antioxidant capacity of Trolox and reference standard solutions of anthocyanins was determined using the $ABTS^{\bullet+}$ scavenging assay. Inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of each compound (Fig. 5). Subsequently, the data were adjusted by linear regression and the slope relation between reference standards and Trolox were used to determine the antioxidant capacity measured as TEAC for individual anthocyanins. A comparison between the antioxidant capacity of the anthocyanins indicated that Dp-3-G had the highest antioxidant capacity (6.99 mM) followed by Pg-3-G (3.68 mM). On the other hand, Mv-3-G had the lowest TEAC value (1.37 mM). According to these TEAC values (mM) and the concentration of the individual anthocyanins (mg/100 g FW), the contribution of the major individual anthocyanins to the TAC value of untreated and CO_2-treated table grapes was calculated (mM TE/100 g FW, Table 3).Our results indicate that after 3 days storage at 0 °C the untreated grapes had the highest anthocyanin content (27.55 mg/100 g FW) and displayed the largest TAC value (52.45 mM TE/100 g FW). Although an increase in each of the anthocyanins contributed to this high TAC value, Pn-3-G was mainly responsible for this sharp early rise in the TAC value of untreated grapes. Pn-3-G had a low average TEAC value (1.73 mM) but its strong contribution could be explained by the drastic increase in the content of this predominant anthocyanin after 3 days in storage at low temperature (Table 3). Conversely, no significant difference was observed in the total TAC value in 3-day CO_2-treated grapes and the Pn-3-G values remained at similar values to those observed in pre-stored grapes. Pg-3-G was present in relatively small amounts in these berries but it had a very high average TEAC value (3.68 mM) and thus, it was an important contributor to the TAC value in CO_2-treated grapes. Additionally, our results indicated that as result of the changes in the concentration of individual anthocyanins, the TAC contribution showed significant changes throughout low-temperature storage (Table 3). The LSD test confirmed that the factors time (D) and time × CO_2-treatment interaction (D × T), significantly affected the TAC contribution of individual anthocyanins and the TAC value of untreated and CO_2-treated table grapes ($P < 0.05$). The highest F value for the factor time was found for the contribu-

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009

Table 3
TAC contribution (mM TE/100 g FW) of the individual anthocyanins and calculated TAC value of untreated and CO_2-treated cardinal table grapes during storage at 0 °C

Days	Pn-3-G		Mv-3-G		Cy-3-G		Pg-3-G		Dp-3-G		TAC value[a]	
	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2
0	23.97 ± 5.79	23.97 ± 5.79	1.65 ± 0.15	1.65 ± 0.15	0.84 ± 0.01	0.84 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	0.95 ± 0.11	0.95 ± 0.11	27.63 ± 6.09	27.63 ± 6.09
3	47.27 ± 0.62	24.53 ± 1.57	1.95 ± 0.20	1.30 ± 0.26	1.25 ± 0.25	1.04 ± 0.12	0.58 ± 0.02	0.72 ± 0.03	1.38 ± 0.03	0.30 ± 0.11	52.45 ± 1.12	27.91 ± 2.10
6	28.15 ± 8.14	22.29 ± 5.59	1.56 ± 0.37	1.31 ± 0.28	0.97 ± 0.17	0.65 ± 0.11	0.40 ± 0.04	0.30 ± 0.05	0.75 ± 0.10	0.21 ± 0.04*	31.84 ± 8.83	24.77 ± 6.08
12	19.34 ± 0.10	33.13 ± 5.91	1.31 ± 0.12	2.30 ± 0.08	0.70 ± 0.03	1.05 ± 0.27	0.29 ± 0.03	0.31 ± 0.05	0.63 ± 0.04	1.11 ± 0.33	22.27 ± 0.34	37.89 ± 6.66
22	24.89 ± 3.37	22.24 ± 1.97	1.48 ± 0.20	1.34 ± 0.33	1.02 ± 0.03	0.61 ± 0.03	0.31 ± 0.04	0.29 ± 0.07	0.92 ± 0.06	0.69 ± 0.06	28.61 ± 3.72	25.18 ± 1.97
33	31.94 ± 6.22	37.59 ± 4.51	1.54 ± 0.37	2.01 ± 0.25	0.93 ± 0.11	1.01 ± 0.19	0.29 ± 0.05	0.33 ± 0.05	0.86 ± 0.36	0.92 ± 0.10	35.55 ± 7.12	41.86 ± 5.11
Significance	D*, D × T*		D*, D × T*		D*, D × T*		D*, D × T*		D*, T*, D × T*		D*, D × T*	

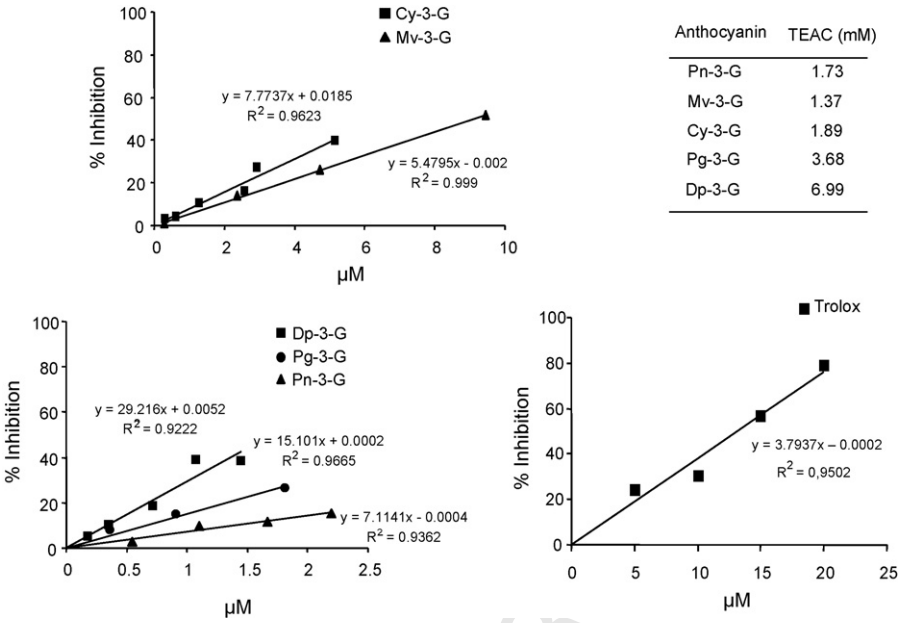
* Significant at $R < 0.05$, where D = days and T = CO_2-treatment.
[a] TAC value was calculated as the sum of the contribution of individual anthocyanins. The contribution of Pt-3-G to the TAC value is not included.

tion of Pg-3-G TAC (F value = 79.49), followed by Mv-3-G (F value = 15.26). The effect of exposure to high CO_2 throughout low-temperature storage was only significant on the TAC contribution of Dp-3-G. The decrease in the levels of Dp-3-G up to 6 days in storage at 0 °C seemed to explain the significant effect of CO_2 on the TAC contribution of this anthocyanin.

4. Discussion

Cold-stressed growth conditions are known to produce an increase in anthocyanin synthesis, although the mechanisms underlying temperature sensitivity in anthocyanin production are poorly understood (see reviews by Mol et al., 1996; Chalker-Scott, 1999). In general, the effects of low-temperature storage on anthocyanin production have been assessed in terms of total anthocyanin levels rather than changes in the levels of specific anthocyanins. In the present work, we have identified the individual anthocyanin pigments in Cardinal table grapes using mass spectrometry and we have examined the effects of low temperature (0 °C) with and without high CO_2 (20%) on each of these. Moreover, instead of relative peak areas, the precise determination of the concentration of the individual anthocyanins in this variety has been calculated. We identified six anthocyanins and in contrast to wine grapes in which the main anthocyanin has been reported to be Mv-3-G (Muñoz-Espada et al., 2004), Pn-3-G was the predominant anthocyanin in the skin of table grapes. It is also important to note the presence of Pg-3-G in the skin of Cardinal table grapes. The presence of 3-*O*-glucoside of pelargonidin was also previously found in Concord, Rubired, and Salvador grape juices. Rubired and Salvador grapes are hybrids from *V. vinifera* and *Vitis rupestris* and Concord is a grape from the native American cultivar *Vitis labrusca* (Wang et al., 2003). The amount of the six anthocyanins found in untreated grapes all increased after storage for 3 days at 0 °C. In accordance with the anthocyanin biosynthetic pathway in plants (Schijlen et al., 2004; Winkel-Shirley, 2001; Stiles et al., 2007), we suggest that the cyanindin, delphinidin and pelargonidin branches seem to be involved in anthocyanin accumulation during the first days of storage at 0 °C. Although further work is needed to know the mechanism implicated in the induction of anthocyanins in grapes at very low temperature, our results indicate that at least one of the temperature control points could be upstream of dihydrokaempferol, where this branching occurs. This is consistent with the induction of *PAL* and *CHS* mRNA in untreated grapes after 3 days at 0 °C (Sanchez-Ballesta et al., 2007), which may lead to increased upstream enzyme activity. In contrast, short-term exposure to high CO_2 levels (20% for 3 days) had no significant effect on anthocyanin content as the observed values were similar to those in pre-stored grapes. Moreover, in grapes treated for 3 days in CO_2 the increase in pelargonidin, an anthocyanin synthesized directly from dihydrokaempferol, was concomitant with the decrease in anthocyanins synthesized directly from dihydromyricetin, namely delphinidin, petunidin and malvidin. It is known that the anthocyanin biosynthesis pathway is controlled in response to different developmental and environmental cues (Holton and Cornish, 1995; Mol et al., 1998). Another challeng-

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009



Anthocyanin	TEAC (mM)
Pn-3-G	1.73
Mv-3-G	1.37
Cy-3-G	1.89
Pg-3-G	3.68
Dp-3-G	6.99

Fig. 5. Antioxidant capacity of Trolox and standard reference anthocyanin solutions using the ABTS$^{\bullet+}$ scavenging assay. The TEAC values (mM) were calculated from the standard and Trolox slope ratios.

ing aspect would be to clarify the detailed course of the reactions and control systems involved in the effects of low temperature and high CO_2 on anthocyanins biosynthesis in fruit tissues.

Anthocyanins are the most prominent pigments in grape skin and they are strong antioxidants. Their double bond conjugate systems allow electron delocalization, resulting in very stable structures and a powerful antioxidant activity. Furthermore, the extent and position of hydroxylation and methoxylation in the B ring modulates their stability and reactivity (Muñoz-Espada et al., 2004). Differences in antioxidant activities between various anthocyanins have been noted in several studies (Tsuda et al., 1994; Wang et al., 1997; Deighton et al., 2000). Moreover, the increase in anthocyanin content in table grapes measured by the pH differential method was not always associated with a similar proportional increase in antioxidant capacity measured by ABTS assay (Sanchez-Ballesta et al., 2007). It has been reported that pH differences have a major influence on scavenging capacity of wine anthocyanins(Borkowski et al., 2005) and that the presence of acid in the solvent has a influence negative in the antioxidant capacity of samples measured by ABTS procedure (Pérz-Jiménez and Saura-Calixto, 2006). In the present work we decided to calculate the TAC value of the samples considering the contribution of individual anthocyanins to the TAC on the basis of their concentration and antioxidant capacity. We found that the calculated TAC values were higher in untreated grapes stored for 3 days at 0 °C than in pre-stored grapes. The rise in Pn-3-G levels, the predominant anthocyanin in this variety, contributed to the peaking of TAC value in grapes at the beginning of low-temperature storage. Moreover, the fact that the calculated TAC values were lower in grapes treated with CO_2 seems to be due to the maintenance of Pn-3-G levels.

We suggest that the accumulation of anthocyanins at the beginning of storage at 0 °C may be a response to a burst in free radicals originated by the fruits' own metabolism in response to the temperature shift. At this low temperature, the untreated fruit triggers this natural antioxidant defense mechanism to reduce the severity of stress. Since the calculated antioxidant capacity in our CO_2-treated table grapes was similar to that found in pre-stored grapes, we can conclude that CO_2 reduces the sensitivity of these grapes to low temperature rather than activating this defense mechanism. In this sense, we previously reported that the effectiveness of this gaseous treatment was not mediated by the induction of either *STS* gene expression (Sanchez-Ballesta et al., 2006) or that of PR genes (Romero et al., 2006). Indeed, a decrease or no change in antioxidant activity was reported in fruits stored at low temperature under controlled atmospheric conditions (Van der Sluis et al., 2001). Our results indicate that after prolonged low-temperature storage similar calculated TAC values were obtained from untreated grapes and those exposed to short-term high CO_2.

The colour of red and black grapes results from the accumulation of the corresponding pigments, the orange to red pelargonidin, the red to magenta cyanidin, and the violet to blue delphinidin. Each variety of grapes has a unique set of anthocyanins (Mazza and Miniati, 1993) and the external colour of the skin of the grapes is used to classify grape varieties into the following groups: green-yellow, pink, red, red-grey, red-dark violet, red-black and blue-black. The mature berries of cardinal table grapes usually possess red or violet tones of greater or lesser intensity, and the amount and nature of its anthocyanins could situate this variety in the red-black group (Carreño et al., 1997). These authors also reported that the L^* hue angle and chroma values in grapes with violet tints were closely correlated, both between each other and also with the visual color, while the value of a^* was not a representative parameter of color. Taking into account the results obtained here, it seems that the most significant differences in the L, chroma and hue angle values between untreated and CO_2-treated grapes appear during the

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009

initial days of storage at 0 °C. The fact that the reduction in the blue tones was greater in CO_2-treated than in untreated grapes in the first 6 days of storage at 0 °C could be associated with the decrease in the amount of delphinidin pigments. However, the possible relationship between colour and the changes in the levels of Dp-3-G and Pg-3-G after exposure to high CO_2 levels should be further studied. After 22 days storage at 0 °C, the colour of untreated and CO_2-treated berries becomes dark violet with red-violet tints of lesser intensity. At this time, the L^*, b^*, chroma and hue anglue values are in accordance with those reported by Carreño et al. (1997) for ripe dark violet grapes.

Until now, the presence of anthocyanins characteristic of any particular species has been useful in testing or confirming the interspecies origin of the genotypes that produce the characteristics of grapes and blackberries. The data we have obtained provide a new framework for the effect of high CO_2 treatment and low temperature on the induction of specific anthocyanins in table grapes. Furthermore, the present results show that the analysis of the profiles of individual anthocyanins and their contribution to the TAC value represents a valuable tool to define their tolerance to specific environmental storage conditions.

Acknowledgements

This work was supported by research grants AGL2005-04502/ALI from CICYT (Spain). M.T.S.-B. and R.M. were supported by "Ramon y Cajal" postdoctoral contracts from the MEC and an I3P fellowship from the CSIC (Spain), respectively. The authors are grateful to Agrotecnica for industrial assistance.

References

Awika, J.M., Rooney, L.W., Waniska, R.D., 2004. Anthocyanins from black sorghum and their antioxidant properties. Food Chem. 90, 293–301.

Borkowski, T., Szymusiak, H., Gliszczynska-Swiglo, A., Rietjens, I.M.C.M., Tyrakowska, B., 2005. Radical scavenging capacity of wine anthocyanins is strongly pH-dependent. J. Agric. Food Chem. 53, 5526–5534.

Carreño, J., Almela, L., Martínez, A., Fernández-López, J.A., 1997. Chemataxonomical classification of red table grapes based on anthocyanin profile and external colour. Lebensm. Wiss. U. Technol. 30, 259–265.

Chalker-Scott, L., 1999. Environmental significance of anthocyanins in plant stress responses. Photochem. Photobiol. 70 (1), 1–9.

Christie, P.J., Alfenito, M.R., Walbot, V., 1994. Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta 194, 541–549.

De Beer, D., Joubert, E., Marais, J., Manley, M., 2006. Unravelling the total antioxidant capacity of Pinotage wines. Contribution of phenolic compounds. J. Agric. Food Chem. 54, 2897–2905.

De Pascual-Teresa, S., Santos-Buelga, C., Riva-Gonzalo, J.C., 2002. LC–MS analysis of anthocyanins from purple corn cob. J. Sci. Food Agric. 82, 1003–1006.

Deighton, N., Brennan, R., Finn, C., Davies, H.V., 2000. Antioxidant properties of domesticated and wild *Rubus* species. J. Sci. Food Agric. 80, 1307–1313.

Faragher, J.D., 1983. Temperature regulation of anthocyanin accumulation in apple skin. J. Exp. Bot. 34, 1291–1298.

Harbone, J.B., Grayer, R.J., 1988. The anthocyanins. In: Harbone, J.B. (Ed.), The Flavonoids: Advances in Research, Since 1980. Chapman and Hall, New York.

Holton, T.A., Cornish, E.C., 1995. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7, 1071–1083.

Hong, V., Wrolstad, R., 1990. Characterization of anthocyanin containing colorants and fruits juices by HPLC/photodiode array detection. J. Agric. Food Chem. 38, 708.

Mazza, G., Miniati, E., 1993. In: Mazza, G., Miniati, E. (Eds.), Anthocyanins in Fruits, Vegetables and Grains. CRC Press, Boca Raton, FL, pp. 149–199.

Mol, J., Jenkins, G.I., Schäfer, E., Weiss, D., 1996. Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis. Crit. Rev. Plant Sci. 15, 525–557.

Mol, J., Grotewold, E., Koes, R., 1998. How genes paint flowers and seeds. Trends Plant Sci. 3, 212–217.

Muñoz-Espada, A.C., Wood, K.V., Bordelon, B., Watkins, B.A., 2004. Anthocyanin quantification and radical scavenging capacity of Concord, Norton, and Marechal Foch grapes and wines. J. Agric. Food Chem. 52, 6779–6786.

Oren-Shamir, M., Levi-Nissim, A., 1997. Temperature effect on the leaf pigmentation of *Cotinus coggygria* 'royal purple'. J. Hortic. Sci. Biotechnol. 72, 425–432.

Pérz-Jiménez, J., Saura-Calixto, F., 2006. Effect of solvent and certain food constituents on different antioxidant capacity assays. Food Res. Int. 39, 791–800.

Re, R., Pelligrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237.

Remon, S., Ferrer, A., López-Buesa, P., Oria, R., 2004. Atmosphere composition effects on Burlat cherry colour during cold storage. J. Sci. Food Agric. 84, 140–146.

Romero, I., Sanchez-Ballesta, M.T., Maldonado, R., Escribano, M.I., Merodio, C., 2006. Expression of class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO_2 pretreatment. Postharvest Biol. Technol. 41, 9–15.

Sanchez-Ballesta, M.T., Bernardo-Jiménez, J., Romero, I., Orea, J.M., Maldonado, R., González-Ureña, A., Escribano, M.I., Merodio, C., 2006. Effect of high CO_2 pretreatment on quality, fungal decay and molecular regulation of stilbene phytoalexin biosynthesis in stored table grape. Postharvest Biol. Technol. 42, 209–216.

Sanchez-Ballesta, M.T., Romero, I., Bernardo-Jiménez, J., Orea, J.M., González-Ureña, A., Escribano, M.I., Merodio, C., 2007. Involvement of the phenylpropanoid pathway in the response of table grapes to low temperature and high CO_2 levels. Postharvest Biol. Technol., doi:10.1016/j.postharvbio.2007.04.001.

Schijlen, E.G., Ric de Vos, C.H., van Tunen, A.J., Bovy, A.G., 2004. Modification of flavonoid biosynthesis in crops plants. Phytochemistry 65 (19), 2631–2648.

Shichijo, C., Hamada, T., Hiraoka, M., Jonhson, C.B., Hashimoto, T., 1993. Enhancement of red-light-induced anthocyanin synthesis in sorghum first internotes by moderate low temperature given the pre-irradiation culture period. Planta 191, 238–245.

Solomon, A., Golubowicz, S., Yablowicz, Z., Grossman, S., Bergman, M., Gottlieb, H.E., Altman, A., Kerem, Z., Flaishman, M.A., 2006. Antioxidant activities and anthocyanin content of fresh fruits of common fig (*Ficus carica* L.). J. Agric. Food Chem. 54, 7717–7723.

Stiles, E.A., Cech, N.B., Dee, S.M., Lacey, E.P., 2007. Temperature-sensitive anthocyanins production in flowers of *Plantago lanceolata*. Physiol. Plant. 129, 756–765.

Tsuda, T., Watanabe, M., Ohshima, K., 1994. Antioxidative activity of the anthocyanin pigments cyanidin 3-*O*-beta-D-glucoside and cyanidin. J. Agric. Food Chem. 42 (11), 2407–2410.

Van der Sluis, A.A., Dekker, M., de Jager, A., Jongen, W.M.F., 2001. Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. J. Agric. Food Chem. 49, 3606–3613.

Wang, H., Cao, G.H., Prior, R.L., 1997. Oxygen radical absorbing capacity of anthocyanins. J. Agric. Food Chem. 45 (2), 304–309.

Wang, H., Race, E.J., Shrikhande, A.J., 2003. Characterization of anthocyanins in grape juices by ion trap liquid chromatography–mass spectrometry. J. Agric. Food Chem. 51, 1839–1844.

Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology and biotechnology. Plant Physiol. 126 (2), 485–493.

Please cite this article in press as: Romero, I., et al., Individual anthocyanins and their contribution to total antioxidant capacity in response to low-temperature storage and high CO_2 in cardinal table grapes, Postharvest Biol. Technol. (2008), doi:10.1016/j.postharvbio.2007.11.009

Discusión

1. Evolución de la calidad de uvas conservadas con altos niveles de CO_2 y bajas temperaturas.

Uno de los objetivos de este trabajo ha sido estudiar la eficacia del pretratamiento con altas concentraciones de CO_2 en el mantenimiento de la calidad de los diferentes tejidos de uva durante su conservación a bajas temperaturas (0ºC). Los parámetros de calidad se analizaron a lo largo del periodo de conservación en frutos tratados y no tratados, coincidiendo con el muestreo realizado al finalizar el tratamiento gaseoso (3 días con 20% CO_2) y durante su posterior transferencia al aire.

1.1. Parámetros de calidad de la baya.

La aplicación de altos niveles de CO_2 durante 3 días a bajas temperaturas no modificó el contenido de sólidos solubles (SSC) ni el pH del zumo, observándose valores similares a los cuantificados en los frutos recién cosechados. Por el contrario en los frutos no tratados y mantenidos en aire durante 3 días a 0ºC se observó un aumento significativo en los valores de SSC y pH, coincidiendo con una disminución en la acidez titulable (TA). Después de 6 días de conservación estas diferencias en los parámetros de calidad entre uvas tratadas y no tratadas fueron menos notables. Asimismo, al final del periodo de conservación no se observaron diferencias significativas en cuanto al contenido de SSC entre uvas tratadas y no tratadas, aunque en ambos casos aumentó significativamente con respecto a los frutos recién cosechados. Estos cambios en el contenido de SSC y TA por efecto de la interacción bajas temperaturas-alto CO_2 se vieron reflejados en la evolución del índice de madurez (SSC/TA), el cual fue menor en uvas tratadas con CO_2, indicando que el tratamiento gaseoso redujo los cambios metabólicos durante la conservación. Se ha descrito que los valores de SSC, TA y pH permanecen constantes en distintas variedades de uva almacenadas a 0ºC bajo diferentes CA (Crisosto et al., 2002; Artés-Hernández et

al., 2006). Resultados similares se obtuvieron en uvas Muscadine almacenadas durante 24 días a 0, 4,5 y 20ºC (Takeda et al., 1983). Sin embargo, en uvas de mesa Flame se observó un incremento en el contenido de SSC de 2,5 ºBrix después de 4 días a 1ºC, mientras que en uvas almacenadas bajo condiciones de MAP durante el mismo periodo de tiempo el incremento en los valores de SSC fue de 1 ºBrix (Martínez-Romero et al., 2003). Respecto a las modificaciones de la textura durante los primeros días de conservación no se observaron diferencias significativas en la firmeza de las uvas tratadas con alto CO_2 con respecto a los frutos recién cosechados. En trabajos previos se ha descrito la eficacia de las altas concentraciones de CO_2 en el mantenimiento de la firmeza de la chirimoya durante el proceso de maduración a 20ºC (Del Cura et al., 1996), y de la adhesión celular durante la conservación a temperaturas de daño por frío (Maldonado et al., 2002). En tomates pretratados con altos niveles de CO_2 también se observó un mantenimiento de la firmeza debido al efecto del CO_2 bloqueando la acumulación de mensajeros de genes implicados en los principales cambios de la maduración, entre los que se encuentra el gen de la poligalacturonasa (Rothan et al., 1997).

Respecto al color, comparando los frutos tratados y no tratados, los cambios más notables en los parámetros *L**, croma y ángulo hue se observaron al finalizar el tratamiento gaseoso de 3 días. Concretamente en los frutos tratados con CO_2 se observó un menor descenso en los valores de los parámetros de color analizados y un incremento en el ángulo hue. El valor de croma en la piel de las bayas tratadas y no tratadas disminuyó progresivamente durante el periodo de conservación alcanzándose valores mínimos después de 22 días de conservación. Se ha descrito previamente que la disminución en los parámetros implicados en el color, cuando el color visual de las uvas era más intenso, podría ser atribuida al hecho de que las uvas pasaban a ser más oscuras al adquirir una coloración violeta oscuro (Carreño y Martínez, 1995). Resultados similares en el color han

sido descritos en uvas de mesa cv. Autummn seedless tras 60 días de conservación en CA (5 kPA de O_2+15 kPA de CO_2) a 0ºC (Artés-Hernández et al., 2004). Distintos estudios demuestran la importancia del color como indicador de la calidad, y por tanto, de la aceptabilidad del fruto (Clydesdale, 1993). Según Carreño et al. (1997) las uvas de mesa cv. Cardinal podrían estar incluidas en el grupo de las uvas rojas-negras por la cantidad total de antocianos y naturaleza del antociano mayoritario. Además de la variedad, las condiciones agronómicas y culturales influyen en los perfiles de antocianos afectando al color de la uva (Mazza y Maniati, 1993). En este trabajo se han observado ligeras variaciones en el valor del ángulo hue, uno de los parámetros más representativos de la apariencia visual de la piel de uvas de esta variedad, tanto en frutos tratados como no tratados con CO_2 que podrían estar relacionadas con los cambios en el perfil de antocianos analizados.

Por último, no se observó oscurecimiento en la pulpa de la uva ni se apreciaron olores ni sabores anómalos, que podrían haberse producido como consecuencia de posibles procesos fermentativos a lo largo del periodo de conservación. Estos resultados son muy positivos, ya que se han observado este tipo de efectos indeseables desde el inicio de la aplicación de CA en distintos frutos (Uota, 1957; Crisosto et al., 2002,b; Kader, 2002).

1.2. Parámetros de calidad asociados al racimo.

El oscurecimiento y marchitamiento del raquis influyen en la apariencia visual de los racimos y ambos parámetros pueden llegar a determinar el periodo máximo de conservación de la uva de mesa. Se ha descrito que distintas concentraciones de CO_2, independientemente de los niveles de O_2, afectan al oscurecimiento del raquis. Así, tras un periodo de conservación de 1 a 2 meses utilizando concentraciones de CO_2 de 10 kPa se observó un mayor oscurecimiento del raquis en las uvas Redglobe cosechadas

tempranamente, mientras que las cosechadas tardíamente toleraron hasta 15 kPa de CO_2 antes de comenzar a mostrar oscurecimiento del raquis en el segundo mes de almacenamiento (Crisosto et al., 2002). En los ensayos realizados en este trabajo se ha observado que los racimos de uva cv. Cardinal toleraron pretratamientos de hasta 20 kPa sin mostrar síntomas de oscurecimiento a los 33 días de conservación. Por el contrario, en los racimos almacenados en aire durante 33 días a 0ºC se observó un considerable deterioro en la apariencia visual de los mismos. El efecto beneficioso del pretratamiento con CO_2 en la apariencia de los racimos podría ser explicado por la menor pérdida de agua observada en el raquis de las uvas sometidas a este tratamiento. Se midió el contenido relativo de agua (RWC, del inglés *Relative Water Content*) durante el periodo de conservación y se observó que este parámetro disminuyó significativamente en el raquis de los racimos no tratados como resultado del almacenamiento a 0ºC. Distintas especies de plantas sometidas a condiciones de estrés hídrico muestran una disminución en el RWC (Nagy et al., 1995; Walter et al., 1990) que parece estar relacionado con la actividad metabólica del tejido (Flower and Lidlow, 1986). Asimismo, se observó que la pérdida de peso en los racimos de uvas tratadas con CO_2 fue menor que en uvas no tratadas después de 33 días de almacenamiento (3,93% y 7,42% respectivamente). Esta menor pérdida de peso observada en los racimos tratados con CO_2 podría indicar una menor actividad metabólica de los mismos bajo el tratamiento gaseoso, coincidiendo con un retraso del proceso de envejecimiento del raquis. En este sentido se ha observado que tejidos fotosintéticos de frutos tratados con alto CO_2 mantienen mejor su maquinaria fotosintética, al evitar la degradación de clorofilas totales y la pérdida de proteínas solubles del cloroplasto (Merodio et al., 1998).

Por tanto, a la vista de estos resultados, se podría concluir que el pretratamiento de 3 días con altos niveles de CO_2 es adecuado para mantener la calidad de la uva de mesa.

Los resultados obtenidos en relación a este mantenimiento son similares a los descritos para diferentes variedades de uva tratadas con atmósferas ricas en CO_2 (Crisosto et al., 2002; Artés-Hernández et al., 2004). En estudios previos, Yahia et al. (1983) así como Berry y Aked (1997) ya describieron distintos efectos beneficios del tratamiento de uvas con CO_2, tales como retraso de la senescencia, disminución de la respiración de la baya y del raquis, reducción del oscurecimiento del raquis, mantenimiento de la firmeza de la baya, y retraso de la podredumbre. Sin embargo, la formación de sabores anómalos y el oscurecimiento de la baya fueron problemas derivados del tratamiento gaseoso (Kader, 2002).

2. Aislamiento y caracterización de los genes que codifican las principales enzimas implicadas en la ruta de los fenilpropanoides (PAL, STS, CHS).

Con el fin de estudiar el efecto del pretratamiento con altos niveles de CO_2 así como las bajas temperaturas de conservación sobre enzimas clave de la ruta de los fenilpropanoides (PAL, CHS y STS), se aislaron cDNAs parciales de las mismas a partir de la piel de uvas de mesa.

El cDNA parcial *VcPAL* codificó un polipéptido de 222 aminoácidos (aa) que presentó elevados niveles de identidad con la secuencia de *V. vinifera* cv. Lambrusco (Sparvioli et al., 1994) y con otras especies de plantas. El cDNA parcial *VcCHS* de 634 pares de bases (pb) codificó un polipéptido de 211 aa y presentó más del 90% de identidad con tres CHS de distintas variedades de *Vitis* depositadas en las bases de datos, mostrando mayor homología con CHS2 de *V. vinifera* cv. Cabernet Sauvignon (Goto-Yamamoto et al., 2002). Por otro lado, la secuencia parcial del cDNA *VcSTS*, de 773 pb, codificó un polipéptido de 257 aa que compartió niveles significativos de identidad con secuencias de STS de *Vitis* (Melchior and Kindl, 1990; Wiese et al., 1994).

La conservación a 0ºC durante 3 días incrementó la abundancia de los transcritos *PAL*, *STS* y *CHS* en la piel de uvas no tratadas, pero estos niveles disminuyeron después de 6 días. Al finalizar los 3 días de tratamiento con CO_2 a 0ºC, los niveles de mRNA de *PAL*, *STS* y *CHS* fueron menores que en los frutos no tratados y prácticamente indetectables cuando los frutos se transfirieron al aire a la misma temperatura. En tejidos fotosintéticamente activos, como las hojas de *Arabidopsis thaliana*, los mRNAs de *PAL* y *CHS* se acumularon a bajas temperaturas de manera dependiente de luz (Leyva et al., 1995). Los resultados obtenidos en este trabajo utilizando frutos almacenados en oscuridad, indican que las bajas temperaturas pueden por sí mismas inducir la expresión de estos genes de la ruta de fenilpropanoides en uva. Christie et al. (1994) demostraron que genes de la ruta de biosíntesis de antocianos, como son la *PAL* y *CHS*, pueden ser considerados genes *COR*, regulados por las bajas temperaturas. También se han observado incrementos en la actividad PAL en respuesta a bajas temperaturas en distintas especies (Tan, 1980; Graham y Patersson, 1982). Aunque las uvas cv. Cardinal no mostraron daños durante la conservación a bajas temperaturas, la activación de la expresión de genes de la ruta de los fenilpropanoides los primeros días de almacenamiento a 0ºC podría ser consecuencia de la percepción del fruto de esta baja temperatura, inferior a su óptima de conservación. Asimismo, la atenuación de los cambios en la expresión génica en la fase inicial de la conservación a 0ºC en los frutos tratados con alto CO_2 podría ser debido al efecto protector del pretratamiento gaseoso.

Los niveles de los mRNAs de la *PAL*, *CHS* y *STS* fueron estudiados periódicamente hasta el final de la conservación en frutos tratados y no tratados. El pretratamiento con alto CO_2 redujo el incremento de los transcritos de *PAL* y *STS* observado en uvas no tratadas, y el máximo en la expresión génica se observó a los 28 días de almacenamiento. Sin embargo, la *CHS* presentó una menor acumulación de mensajeros que *PAL* y *STS* tanto en

uvas tratadas como en no tratadas; aunque, al igual que éstas, el máximo se observó a los 28 días y el CO_2 actuó disminuyendo los niveles del transcrito respecto de las uvas mantenidas en aire desde el comienzo de la conservación. La expresión de los genes *PAL* y *CHS* está regulada por distintos estreses entre los que se encuentran, las bajas temperaturas, el ataque por patógenos y el daño mecánico (Leyva et al., 1995; Seki et al., 2002; Sanchez-Ballesta et al., 2000). Se ha considerado que el incremento en la actividad de la enzima PAL, junto con el de otras enzimas involucradas en la biosíntesis de fenilpropanoides, es un mecanismo de defensa que opera en distintas especies vegetales sometidas a distintos estreses (Dixon y Pavía, 1995). En cultivos celulares de *V. vinifera* infectados por *B. cinerea* se observó el descenso en la síntesis de proteínas totales y la acumulación específica de un número pequeño de proteínas involucradas en resistencia inducida, entre las que se encontraban principalmente las enzimas PAL y STS (Liswidowati et al., 1991). En las uvas no tratadas, los mRNAs de *STS*, *PAL* y *CHS* se acumularon principalmente a los 28 días coincidiendo con la mayor infección por hongo de las uvas. La inducción de estilbenos en respuesta al ataque por patógenos ha sido descrita previamente (Sarig et al., 1997). Los estilbenos son compuestos activos biológicamente con actividad antifúngica frente a distintos patógenos de plantas entre los que se encuentra *B. cinerea* (Adrian et al., 1998). Sin embargo, el pretratamiento con altos niveles de CO_2 parece tener un claro efecto inhibidor sobre la inducción de la expresión del gen *STS* durante la conservación de la uva de mesa. Así, el ligero aumento de los niveles del transcrito *STS* observado en los frutos tratados respecto a los niveles encontrados en las uvas recién traídas de campo, se mantuvo estable hasta el final del almacenamiento y fue, en todo caso, menor que el observado en uvas no tratadas, al igual que el porcentaje de ataque por hongos. Estos resultados indican que la eficacia del pretratamiento con CO_2

reduciendo el ataque por hongo durante la conservación a bajas temperaturas parece no estar mediada por la inducción de la expresión de los genes *PAL*, *STS* y *CHS*.

3. Contenido de resveratrol en los extractos de piel de uva.

La concentración de resveratrol en las uvas es muy dependiente del cultivar. En uva de mesa cv. Cardinal, los cambios observados en los niveles de mRNA *STS* fueron paralelos al contenido de *trans*-resveratrol al final del periodo de conservación, demostrando que la acumulación de estilbenos en la piel de las bayas está altamente regulada a nivel de la expresión génica de *STS*. Además, en frutos no tratados se observó que la acumulación de *trans*-resveratrol acompañó al incremento en el porcentaje de ataque por hongos observado al final de la conservación a 0ºC. La producción de fitoalexinas de la familia del estilbeno, es una de las rutas de defensa más importantes en uva (Langcake y Pryce, 1977). Se ha descrito que el resveratrol se acumula en respuesta a estreses bióticos y/o abióticos como: infección por patógenos, radiación UV, o sustancias químicas (Langcake, 1981; Creasy y Coffee, 1988; Jeandet et al., 1995; Sarig et al., 1997; Cantos et al., 2002). Desde que se describió que *B. cinerea* puede actuar como elicitor de la producción de *trans*-resveratrol en vid (Langcake y Pryce, 1976), distintas investigaciones se han dirigido hacia la interacción de este huésped-patógeno (Jeandet et al., 1995; Adrian et al., 1998; Breuil., 1998). Además, esta fitoalexina también ha recibido en los últimos años mucha atención por su implicación en la salud humana (Jang et al., 1997; Hung et al., 2000; Whitsett y Lamortiniere, 2006).

Por otro lado, durante la primera fase de conservación a 0ºC se observó un descenso de los niveles de *trans*-resveratrol, que fue mayor en los frutos tratados con CO_2 al finalizar los 3 días de tratamiento. Es posible que la disminución en los niveles de *trans*-resveratrol fuese el resultado de la unión de subsecuentes moléculas de *trans*-resveratrol

oxidadas dando lugar a la formación de distintos oligómeros, tales como ε y α-viniferinas (Langcake y Pryce, 1977); pero, hasta el momento, se desconoce la causa de la misma. Tras la transferencia al aire de los frutos tratados con altos niveles de CO_2, se observó un aumento paulatino del contenido de *trans*-resveratrol hasta los 33 días de conservación, alcanzando niveles similares a los cuantificados en los frutos recién cosechados.

Durante el periodo de vida útil, cuando las uvas son transferidas a 20ºC, el aumento en el contenido de *trans*-resveratrol fue de un 72% en las uvas tratadas, mientras que sólo aumentó un 10% en las no tratadas. El alto potencial para sintetizar *trans*-resveratrol de las uvas tratadas con CO_2 durante el periodo de vida útil fue similar al de los frutos no tratados al final del almacenamiento a 0ºC. También se ha descrito previamente, un aumento en el contenido de *trans*-resveratrol durante el periodo de vida útil de uvas de mesa almacenadas en CA (Artés-Hernández et al., 2003). Este mayor incremento en los niveles de *trans*-resveratrol coincide con el mayor porcentaje de ataque por hongo, corroborando, una vez más, la relación existente entre la podredumbre y el contenido en *trans*-resveratrol.

4. Antocianos totales y capacidad antioxidante de la piel de uva de mesa conservada a 0ºC y altos niveles de CO_2.

Se analizó la evolución del contenido de antocianos a lo largo de la conservación de las uvas tratadas y no tratadas, así como la capacidad antioxidante de la piel de las mismas, donde se encuentran principalmente estos compuestos.

En los frutos no tratados se observó un incremento transitorio en el contenido de antocianos totales los primeros días de conservación a 0ºC. La aplicación de un 20% de CO_2 evitó este incremento inicial en el contenido de antocianos totales, y únicamente después de la transferencia al aire se alcanzaron valores similares a los cuantificados en frutos no tratados. A lo largo de los muestreos realizados durante la conservación, se

observó una disminución en el nivel de antocianos totales, tanto en uvas tratadas como no tratadas, respecto de los valores de los primeros días de almacenamiento; siendo en todo caso estos niveles superiores a los observados en los frutos recién traídos de campo. Se sabe que la síntesis de antocianos continúa después de la cosecha y también durante la conservación a bajas temperaturas (Kacperska, 1989; Christie et al., 1994; Solecka et al., 1999). Además, Roubelakis-Angelakis y Kliewer (1986) observaron un aumento en la actividad PAL paralela a la acumulación de antocianos durante la maduración de uvas de mesa cv. Cardinal. También se ha descrito un aumento del metabolismo fenólico y de la capacidad antioxidante en espinacas como respuesta a estreses bióticos y abióticos (Howard et al., 2002). Durante los primeros días de almacenamiento se observó un aumento de la capacidad antioxidante, determinada por el método ABTS, en uvas no tratadas que coincidió con el incremento en el contenido de antocianos totales citado anteriormente, aunque no en la misma proporción. Así, mientras que el aumento de la capacidad antioxidante fue de un 20%, los antocianos aumentaron hasta un 62%. Por otro lado, en los frutos tratados con alto CO_2 el ligero incremento en el contenido de antocianos observado al finalizar el tratamiento gaseoso fue asociado a un descenso de un 30% en la capacidad antioxidante respecto de los frutos recién traídos de campo. Este efecto se mantuvo tras la transferencia al aire de la uva, aunque a medida que se perdía el efecto del CO_2 la capacidad antioxidante fue aumentando. También se ha descrito una disminución de la capacidad antioxidante de espinacas frescas cortadas almacenadas a 10ºC en MAP (Gil et al., 1999). Al final de la conservación, la capacidad antioxidante de las uvas no tratadas disminuyó mientras que las tratadas con CO_2 mantuvieron valores similares a los frutos recién traídos de campo. Algunos autores, como Kalt et al. (1999) y Wang y Lin (2000) encontraron una correlación significativa entre la capacidad antioxidante, fenoles totales y antocianos. Por otro lado, algunas investigaciones han indicado que los antocianos

podrían ser menos significativos en la correlación con las propiedades antioxidantes de los frutos (Arnous et al., 2002; Kallithraka et al., 2005). Orak et al. (2007), analizaron las correlaciones existentes entre capacidad antioxidante, fenoles totales y antocianos en distintos cultivares de uva tinta y concluyeron que la capacidad antioxidante determinada en estas bayas no siempre está relacionada con el contenido en antocianos.

5. Identificación y cuantificación de los principales antocianos presentes en la piel de uva. Capacidad antioxidante de los antocianos cuantificados.

Se identificaron y cuantificaron mediante HPLC-DAD-MS seis antocianos monoglucósidos, incluyendo la pelargonidina-3-G. Aunque se había identificado la presencia de pelargonidina-3-G en zumos de uva obtenidos de los cultivares híbridos de *V. labrusca* y *V. rupestris* (Wang et al., 2003), es la primera vez que se describe la presencia de este antociano en frutos de *V. vinifera* contrariamente a lo observado por Carreño et al., (1997). Por otro lado y coincidiendo con estos autores, la peonidina-3-G es el antociano predominante en la piel de uva de mesa cv. Cardinal. La conservación a 0ºC durante los 3 primeros días incrementó la concentración de cada una de los seis antocianos identificados. Es importante destacar el gran incremento en el contenido de peonidina-3-G (+97%), alcanzando valores de 25,20 mg/100 g peso fresco y de pelargonidina-3-G (+141%), que pasó de 0,061 a 0,147 mg/100 g peso fresco. El incremento fue menos pronunciado en cianidina-3-G (+49%), delfinidina-3-G (+48%) y petunidina-3-G (+25%), siendo moderado en malvidina-3-G (+18%). De acuerdo con la ruta biosintética de antocianos en plantas (Schijlen et al., 2004, Winkel-Shirley, 2001; Stiles et al., 2007), y considerando los cambios mencionados en los antocianos identificados en esta variedad, se sugiere que, en los frutos después de 3 días de almacenamiento en aire a 0ºC, se activan las tres vías de síntesis de los antocianos derivados de la cianidina, delfinidina y pelargonidina. Estos

resultados apuntan a que, al menos un punto de control de la temperatura en la ruta de biosíntesis de antocianos está por encima del punto de ramificación. Esto es consistente con la inducción de los mRNAs de *PAL* y *CHS* observada en uvas no tratadas después de 3 días a 0ºC, lo que podría conducir a un aumento de las actividades enzimáticas antes del punto de control de temperatura previamente citado.

Mientras que el contenido de antocianos en los frutos no tratados alcanzó su máximo (27,55 mg/100 g peso fresco) después de 3 días a 0ºC, al finalizar el tratamiento gaseoso los niveles fueron similares a los de los frutos recién cosechados. Además, la exposición durante 3 días a un 20% de CO_2 no modificó el contenido de peonidina-3-G (0%); incrementó el de cianidina-3-G (+24%) y pelargonidina-3-G (+200%), al tiempo que descendió el de delfinidina-3-G (-64%), malvidina-3-G (-21%) y petunidina -3-G (-27%). Estos resultados apuntan hacía un diferente control del alto CO_2 y las bajas temperaturas sobre la ruta de síntesis de los antocianos.

Los resultados sobre el efecto de la temperatura y el CO_2 en la concentración y perfiles de los antocianos en uva de mesa durante su conservación postcosecha, son acordes con los descritos previamente por Holton y Cornisa (1995) y Mol et al. (1998); donde se indica que la ruta de biosíntesis de los antocianos es controlada en respuesta a diferentes estreses ambientales así como durante la etapa de crecimiento y desarrollo del fruto. Al final de la conservación, los valores de los antocianos fueron, en general, superiores a los obtenidos en los frutos recién traídos de campo, siendo la peonidina-3-G la que presentó el mayor aumento a los 33 días de conservación; alrededor de un 44% en frutos no tratados y de un 70% en frutos tratados. Sin embargo, las excepciones fueron la delfinidina-3-G y la malvidina-3-G que no aumentaron y mantuvieron valores similares a los de los frutos recién cosechados.

En base a la aparente falta de unanimidad en la correlación entre los valores de capacidad antioxidante y contenido en antocianos totales, se planteó el cálculo de la capacidad antioxidante total (TAC) de los frutos tratados y no tratados a lo largo de la conservación, teniendo en cuenta la concentración de cada antociano presente en los extractos y su correspondiente capacidad antioxidante. Asimismo, considerando las grandes diferencias en cuanto a los valores de capacidad antioxidante asignados a cada antociano presentes en la bibliografía (Wang et al., 1997; Deighton et al., 2000; Wang y Lewers, 2007), se optó por analizar la capacidad antioxidante de los diferentes antocianos identificados en nuestra variedad a partir del estudio con patrones comerciales de antocianos. Además, para una mayor precisión, la capacidad antioxidante de cada antociano se calculó según el método ABTS utilizando rectas de calibrado realizadas con diferentes concentraciones de patrones. Los resultados obtenidos indicaron que el antociano con mayor capacidad antioxidante, calculado en equivalentes de Trolox (TEAC), fue la delfinidina-3-G (6,99 mM), seguida de la pelargonidina-3-G (3,68 mM), cianidina-3-G (1,89 mM), peonidina-3-G (1,73 mM) y malvidina-3-G (1,37 mM). Estos datos difieren de los observados por Wang et al. (1997), donde se describe que la cianidina-3-G es el antociano con mayor capacidad antioxidante. El estudio comparativo de los valores de TAC entre frutos tratados y no tratados durante su conservación indicó que el valor máximo (52,45 mM TE/100 g peso fresco) lo exhibieron los frutos no tratados después de 3 días a 0 ºC. La peonidina-3-G fue la responsable principal de este incremento en el valor de TAC calculado. Aunque el valor de TEAC para este antociano resultó ser intermedio (1,73 mM), su contribución a la TAC podría ser explicada por el importante aumento de su contenido en uvas no tratadas al principio de la conservación. Sin embargo, en las uvas tratadas con CO_2, tanto los valores de peonidina-3-G como de la TAC fueron similares a los encontrados en uvas recién cosechadas. La transitoriedad de los efectos de las bajas temperaturas y el alto CO_2 dio lugar a que las

diferencias en los valores de TAC entre uvas tratadas y no tratadas no fueran significativas al final de la conservación. Además, el estudio comparativo de la TAC calculada a partir de la suma de capacidad antioxidante de cada uno de los antocianos de la muestra, y de la medida a partir de los extractos, nos confirma la infravaloración de la actividad antioxidante empleando el método ABTS, el cual se ve afectado negativamente por pHs ácidos (Pérez-Jiménez et al., 2006). De este modo, se puede concluir que después de 3 días a 0ºC el incremento transitorio del contenido de antocianos se correspondía con un aumento también transitorio en su capacidad antioxidante. Estos resultados permiten sugerir que el incremento en la capacidad antioxidante de los frutos podría responder a un estallido en la producción de radicales libres originado por el metabolismo del fruto en respuesta a las bajas temperaturas de conservación. A 0ºC, los racimos mantenidos en aire podrían activar su mecanismo de defensa antioxidante para reducir la severidad del estrés causado por los radicales libres. Por el contrario, el mantenimiento de los valores iniciales de TAC en las uvas tratadas con CO_2, permite sugerir que el CO_2 reduce la sensibilidad del fruto a las bajas temperaturas de manera que parece no necesitar activar su capacidad antioxidante. Estas sugerencias se ven apoyadas por los resultados obtenidos de la disminución en la expresión génica de la ascorbato peroxidasa (*APX)* observada en las bayas tratadas con alto CO_2. El cDNA parcial de la *APX*, de 539 pb, se obtuvo por RT-PCR y la secuencia deducida de 179 aa presentó el dominio de unión al grupo hemo que comparten todas las proteínas APX, mostrando una elevada identidad con las secuencias de APX citosólicas (82-98%) depositadas en las bases de datos. En cuanto a sus niveles de expresión, la acumulación del transcrito aumentó ligeramente tanto en las uvas tratadas como en las no tratadas, pero se observó un fuerte aumento después de 28 días de conservación en las uvas no tratadas coincidiendo con el mayor desarrollo de podredumbre. Aunque existen distintos estudios sobre APX citosólicas en diferentes

plantas y frutas (Mittler and Zilinskas, 1991; Ishiwaka et al., 1995; Kim y Chung, 1998), no existen estudios acerca del papel del gen *APX* en uva de mesa. Por tanto, los resultados obtenidos donde se muestran diferencias en los mecanismos que operan en las uvas tratadas y no tratadas, conducen hacia la hipótesis de que la enzima APX podría participar eliminando los altos niveles de H_2O_2 existentes en las uvas no tratadas.

Capítulo 2

Efecto del CO_2 en la mejora de la uva de mesa con respecto al ataque por hongo. PRs.

1. Características generales de proteínas relacionadas con la patogénesis (PRs)

Una de las respuestas más frecuentes en las plantas frente al ataque por patógenos es la acumulación de proteínas PRs. En 1971, Abeles et al. sugirieron que las proteínas β-1,3-glucanasa y quitinasa podían participar en la defensa frente a patógenos fúngicos. Por la misma época, las PRs fueron descritas por primera vez como un conjunto original de proteínas que se acumulaban en las hojas de tabaco en relación con la respuesta hipersensitiva a la infección por el Virus del Mosaico del Tabaco (Van Loon y Van Kammen, 1970). Más tarde, se demostró que estas proteínas también eran inducidas por infecciones bacterianas y fúngicas (Redolfi y Cantisani, 1984). Hay distintas observaciones que sugieren un papel defensivo para las proteínas PR como son: la inducción de genes que codifican PRs y proteínas del SAR en plantas como respuesta a subsecuentes infecciones después de una infección necrótica inicial (Kassanis et al., 1974; Van Loon, 1975; Ward et al., 1991); la ausencia de sustrato para una de las proteínas PR (quitinasa) en plantas, pero su presencia en la pared celular de ciertos hongos; así como la actividad antifúngica de quitinasas *in vitro* (Mauch et al.,1988).

Aún no está claro cómo estas proteínas, de forma aislada o combinadas entre sí, pueden participar en la defensa de las plantas frente a la infección por hongos. Se ha propuesto que estas hidrolasas actúan al menos de dos maneras diferentes: directamente, degradando la pared celular del patógeno o indirectamente, promoviendo la liberación de compuestos de la pared celular (oligosacáridos) que pudieran actuar como elicitores de las reacciones de defensa (Meins et al., 1993; Van Loon et al., 1994; Van Loon et al., 2006).

Actualmente se clasifican en 17 familias funcionales que se supone son inducidas como respuesta a infecciones por patógenos (o en situaciones asociadas a la infección), estímulos ambientales o durante el desarrollo (Boller, 1988; Neuhaus, 1999; Van Loon et al., 2006). Durante los últimos años, muchas investigaciones han centrado su interés en

elucidar sus funciones. Específicamente, la principal atención se ha focalizado en el papel fundamental de diferentes quitinasas y β-1,3-glucanasas en la degradación de quitina y β-1,3-glucanos (Aronson, 1965) presentes en la pared celular de patógenos (Mauch et al. 1988). Sin embargo, sólo unas pocas proteínas PR han sido estudiadas a fondo con respecto a sus funciones específicas durante la patogénesis. Esto es debido a la compleja regulación de estas proteínas, ya que aparecen también durante las etapas de desarrollo, y a que algunos centros catalíticos específicos permanecen sin ser todavía identificados (Colditz et al., 2007).

Se ha demostrado que miembros de al menos cuatro de estas familias, como las β-1,3-glucanasas (PR-2), quitinasas (PR-3), proteínas de unión a quitina (PR-4) y proteínas tipo taumatina (PR-5), tienen actividad antifúngica en bioensayos *in vitro,* apoyando el posible papel de estas proteínas en la defensa de plantas (Kombrink y Somssich, 1997).

2. Quitinasas

La importancia de las investigaciones sobre los mecanismos de defensa en plantas ha derivado en un rápido y constante interés por las quitinasas puesto que fueron las primeras proteínas inducidas por patógeno cuya función ha sido identificada. Su sustrato está presente en la pared celular de muchos hongos, insectos, nemátodos, y principales patógenos de las plantas cultivadas (Neuhaus et al., 1987) y, por tanto, son enzimas ubicuas de los mismos. Hidrolizan el enlace β-1,4 entre los residuos de N-acetilglucosamina de quitina, un polisacárido estructural de la pared celular de muchos hongos y del exoesqueleto de invertebrados. Se dividen en dos categorías: exoquitinasas, con actividad sólo en el extremo no reductor de la cadena de quitina; y endoquitinasas, las cuales hidrolizan las uniones internas β-1,4-glicosídicas. Muchas endoquitinasas de plantas, especialmente aquellas con alto punto isoeléctrico, exhiben una actividad lisozima

o tipo lisozima adicional (Collinge et al., 1993; Schultze et al., 1998; Subroto et al., 1999). Asimismo, en distintas especies de plantas se han encontrado quitinasas ácidas y básicas.

Las quitinasas son enzimas sintetizadas por las plantas en respuesta a estreses bióticos y abióticos como heridas, radiación UV, bajas temperaturas, e invasión de hongos, bacterias y virus (Bowles, 1990; Van Loon et al., 2006). Pueden estar localizadas en distintos tejidos y órganos como semillas (Santos et al., 2004), hojas (Robert et al., 2002), raíces (Lam y Ng 2001), tallos (Lee et al. 2000), flores (Takakura et al., 2000), frutos (Díaz-Perales et al., 1998), tubérculos (Forsyth y Shewry 2002) y bulbos (Yamagami et al., 1998). Son codificadas por un número relativamente pequeño de genes, y la localización de los intrones está bastante conservada. Hasta la actualidad se han encontrado múltiples isoenzimas de quitinasas que han sido divididas en siete clases (I-VII) en base a sus propiedades estructurales (Brunner et al., 1998). Sin embargo, la mayoría de las quitinasas, pertenecen a las primeras cuatro clases (Fig. II)

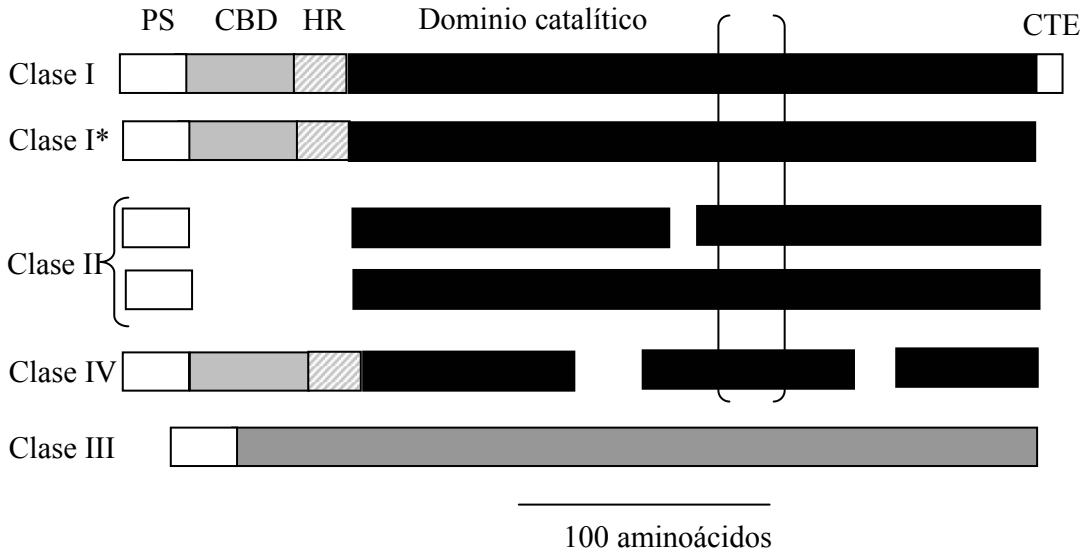


Fig II. Cuadro comparativo de las principales clases de quitinasas (PS: péptido señal; CBD: Dominio de unión a cysteína; HR: región bisagra; CTE: extensión C-terminal)

La familia PR-3 es una familia de quitinasas específica de plantas. Las distintas clases que pertenecen a esta familia han sido definidas primero de acuerdo a la homología de secuencias y a la presencia o ausencia del dominio de unión a quitina (CBD; del inglés *Cysteine-rich chitin-Binding Domain*). Las quitinasas de clase I, II y IV son las más parecidas estructuralmente. Las quitinasas de clase I son sintetizadas como precursores con una secuencia señal N-terminal que las dirige a la ruta secretora. La mayoría de las quitinasas de clase I también presentan un propéptido C-terminal, requerido para su transporte a la vacuola (Neuhaus et al., 1991). La deleción de este péptido señal C-terminal redirige a la quitinasa de clase I al apoplasto pero mantiene la actividad enzimática (Grover et al., 2001). Dentro de la proteína madura, el CBD N-terminal, se une al centro catalítico por un espaciador, variable tanto en longitud como en secuencia, pero a menudo rico en prolina o glicina. En el caso de las quitinasas de tabaco, el espaciador se observó que era modificado por hidroxilación de algunas de las prolinas (Sticher et al., 1992). La función de esta modificación no está clara pero puede ser debido a una semejanza de secuencia accidental entre el espaciador y las glicoproteínas ricas en hidroxiprolina secretadas (Neuhaus et al., 1987). En una quitinasa de clase I de tabaco, la deleción del CBD y de la región espaciadora, por separado o combinadas, redujo la actividad hidrolítica un 50% y la actividad antifúngica en un 80% (Suárez et al., 2001). Las quitinasas de clase II son generalmente ácidas y extracelulares, y pueden ser detectadas en el fluido apoplástico (Benhamou et al., 1990; Dore et al., 1991). Además, no presentan el CBD ni la región bisagra (HR; del inglés *Hinge Region*) presentes en las quitinasas de clase I. Una posible función de estas quitinasas en la defensa de las plantas es actuar como moléculas señal, eliminando los elicitores procedentes de las hifas del hongo que invaden al huésped, y actuando como una primera línea de defensa (Mauch y Staehelin, 1989). Las de clase III (que pertenecen a la familia PR-8) son hidrolasas extracelulares que no se relacionan

estructuralmente con otras quitinasas de plantas ya que poseen un dominio catalítico conservado que difiere del de las quitinasas de clase I o II (Watanabe et al., 1993). Además, la mayoría se clasifican como quitinasas de clase III en base a su identidad con lisozimas previamente descritas con actividad quitinasa (Graham y Sticklen, 1994). Las de clase IV difieren de la clase I por las deleciones internas existentes entre los dominios de unión a quitina y catalítico. Las quitinasas de clases V y VI fueron definidas en base a la presencia de dos CBDs en tandem, o a la presencia de un CBD truncado y a un largo espaciador rico en prolina, respectivamente. Además, las de clase VI son similares a las quitinasas de bacterias como por ejemplo, *Bacillus circulan*s, *Serratia marcescens* o *Streptomyces plicatus* (Melchers et al., 1994). Finalmente, las de clase VII, las últimas detectadas, poseen un dominio catalítico homólogo al de clase IV, pero no presentan el CBD (Punja y Zhang 1993; Neuhaus 1999; Robert et al., 2002).

3. β-1,3-glucanasas

Las β-1,3-glucanasas pertenecen a la familia de proteínas PR-2 y son capaces de catalizar cortes hidrolíticos endógenos en los enlaces β-1,3-glucosídicos de los β-1,3-glucanos, otro de los constituyentes de la pared celular fúngica. Las β-1,3-glucanasas son enzimas muy abundantes, y ampliamente distribuidas en distintas especies de plantas (Meins et al., 1992; Hoj y Fincher, 1995). Aunque el mayor interés radica en el posible papel de las β-1,3-glucanasas en la respuesta de las plantas a patógenos microbianos, hay fuertes evidencias de que estas enzimas también están implicadas en diversos procesos fisiológicos y del desarrollo en plantas no infectadas, incluyendo, división celular, maduración del fruto, respuestas a daño mecánico, bajas temperaturas, ozono y UV-B, entre otros (Waterkeyn, 1967; Fulcher et al., 1976; Hinton y Pressey, 1980; Ernst et al., 1992).

Las β-1,3-glucanasas existen como múltiples isoformas estructurales que difieren en tamaño, punto isoeléctrico, estructura primaria, localización celular, y modelo de regulación (Meins et al, 1992; Robinson et al, 1997). La información acerca de las secuencias de estas isoformas está disponible gracias a los cDNAs y clones genómicos de β-1,3-glucanasa de *Nicotiana tabacum*, que constituyen una familia multigénica. Basándose en la identidad de la secuencia de aminoácidos, las distintas β-1,3-glucanasas de tabaco han sido clasificadas en tres clases estructurales (Meins et al, 1992). Se han descrito isoformas con estructuras similares en tomate, patata, melocotón, plátano y otras especies de plantas (Stone y Clarke, 1992; Thimmapuram et al., 2001; Yamaguchi et al., 2002; Receveur-Bréchot at al., 2006).

Las β-1,3-glucanasas son producidas como preproteínas con un péptido señal N-terminal hidrofóbico, el cual es eliminado co-traduccionalmente, y un extremo C-terminal N-glicosilado en un único sitio. Existen evidencias indirectas de que, análogamente con las quitinasas de clase I de tabaco y la lectina de cebada, el extremo C-terminal contenga señales de envío a la vacuola (Worral et al., 1992). Las proteínas de clase I de tabaco son proteínas básicas localizadas en la vacuola celular. Sin embargo, los miembros de la familia PR-2 de clases II y III son secretados al espacio extracelular (Stintzi et al, 1993; Simmons, 1994). En concreto, estas β-1,3-glucanasas de tabaco son proteínas ácidas sin el extremo C-terminal presente en las enzimas de clase I.

Casi todos los genes de β-1,3-glucanasas aislados de especies de plantas contienen un único intron, con la excepción de un gen aislado de *Nicotiana plumbaginifolia* que contiene dos (Castresana et al., 1990). La posición del intrón es la misma en genes de glucanasas ácidas y básicas, indicando que la estructura de los genes de estas glucanasas

está altamente conservada, y que deben haber surgido de un único ancestro común (Linthorst 1991).

4. Regulación génica de quitinasas y β-1,3-glucanasas

Se ha demostrado la existencia de mensajeros secundarios tales como SA, etileno, JA y óxido nítrico en la ruta de señalización que conduce a la activación de genes que codifican proteínas PR (Grant y Loake, 2000; Glazebrook, 2001; Nurnberger y Scheel, 2001; Wendehenne et al., 2001). El SA parece estar involucrado en la inducción de genes asociados con el desarrollo de la SAR. En *Arabidopsis*, se ha identificado un inhibidor llamado NPR1 (del inglés *non-expressed of PR-genes*) que controla la expresión de genes que codifican PRs y que responde a SA y al ataque por patógenos (Cao et al., 1994; Cao et al., 1997). En hojas de arroz, incrementos en los niveles endógenos de SA dieron como resultado una mayor resistencia frente a *Xanthomonas oryzae* y una reducción de las lesiones de la hoja (Babu et al., 2003). El etileno y el JA parecen actuar en rutas diferentes a las del SA conduciendo a la inducción de genes que codifican proteínas PR básicas, tioninas y defensinas (Feys y Parker, 2000; Glazebrook, 2001).

Los genes de quitinasa y β-1,3-glucanasa están regulados a nivel transcripcional. En judía (*Phaseolus vulgaris*), se observó un incremento en la expresión de *pCH18* que codifica una quitinasa de clase I en respuesta al etileno, elicitores de oligosacáridos y patógenos fúngicos (Broglie et al., 1986). Utilizando una construcción quimérica conteniendo la región promotora del gen β-glucoronidasa, Broglie et al. (1989) mostraron que esta región 5' fue suficiente para la expresión regulada por etileno del gen quitinasa en plantas de tabaco. Asimismo, esta sensibilidad al etileno ha sido estudiada también en el promotor de una quitinasa de tabaco de clase I (Shinshi et al., 1995).

La expresión de β-1,3-glucanasas está también regulada por hormonas o en respuesta al ataque por patógenos. Los niveles de mensajero de β-1,3-glucanasa aumentan en respuesta a la hormona etileno (Hart et al. 1993), y ozono (Thalmair et al., 1996); mientras que disminuyen por tratamiento con ABA (Rezzonico et al. 1998) y por combinación de auxinas y citoquinas (Félix y Meins, 1986; Vögeli-Lange et al., 1994). También son inducidas por infecciones con bacterias (Meins y Ahl, 1989), virus (van de Rhee et al., 1993) y hongos (Chang et al., 1992). Sin embargo, algunos genes de β-1,3-glucanasas están únicamente regulados durante el desarrollo y no son inducidos en respuesta a condiciones de estrés, como el gen de la β-1,3-glucanasa aislado del estilo (Ori et al., 1990) y de la antera (Bucciaglia y Smith, 1994) de tabaco.

Existen evidencias de que proteinas quitinasa y β-1,3-glucanasa, de forma individual y particularmente en combinación, pueden ayudar a defender las plantas contra la infección fúngica. Se han realizado análisis con diferentes isoformas de β-1,3-glucanasas y quitinasas para estudiar la actividad antifúngica *in vitro* de estas proteínas y se ha demostrado que quitinasas purificadas de guisante fueron capaces de inhibir el crecimiento de sólo una especie de hongo, mientras que la combinación de quitinasa y β-1,3-glucanasa inhibió el crecimiento de todos los hongos estudiados mostrando actividades sinérgicas entre estas enzimas (Mauch et al., 1988). A partir de este hallazgo, otros estudios han verificado estos resultados en tabaco (Yun et al. 1996), uvas (Derckel et al., 1998) y arroz (Velazhahan et al., 2000). Esto podría indicar que isoformas específicas son inducidas en respuesta a patógenos concretos y sólo ciertas isoformas son capaces de inhibir hongos específicos (Ji et al., 2000; Sela-Buurlage et al., 1993). Por ejemplo, una quitinasa de clase I de tabaco presentó actividad antifúngica frente *Fusarium solani*; pero las quitinasas de clase II mostraron un ligero efecto en la inhibición del crecimiento del

hongo solamente cuando se usaron junto con altas concentraciones de β-1,3-glucanasas (Jach et al., 1995).

Plantas transgénicas de tabaco que expresan β-1,3-glucanasas, especialmente en combinación con una quitinasa, han mostrado mayor tolerancia a patógenos (Jach et al., 1995; Jongedijk et al., 1995). También plantas transgénicas de trigo que sobreexpresaron un gen de quitinasa de clase II (Bliffeld et al., 1999), presentaron mayor resistencia a patógenos fúngicos. Se ha comprobado que las quitinasas de clase I tienen una mayor actividad antifúngica que el resto de quitinasas quizás debido a la presencia del CBD (Sela-Buurlage et al., 1993). Por otro lado, se ha observado que plantas transgénicas que muestran expresión constitutiva de quitinasas exhiben niveles mayores de resistencia a la infección fúngica y retrasan los síntomas de daño frente a patógenos fúngicos (Broglie et al., 1991; Jach et al., 1995; Grison et al., 1996). Plantas de tabaco transformadas con un gen de quitinasa aislado de judía bajo el control del promotor 35S, presentaron una actividad quitinasa hasta 20 veces superior que las plantas control (Broglie et al., 1991).

En 2001, Porat et al. aislaron un cDNA de quitinasa básica de naranja, y comprobaron que su expresión formaba parte de los mecanismos moleculares implicados en la inducción de resistencia del fruto frente a patógenos. Distintas evidencias apoyan el papel antifúngico de las quitinasas de clase I, IV y VI ya que se ha demostrado la habilidad de las mismas para inhibir el crecimiento de las hifas de los hongos *in vitro* (Collinge et al., 1993; Derckel et al., 1998) o *in vivo* en el caso de quitinasas de clase I (Benhamou et al., 1993).

Durante la aclimatación al frío, algunas plantas, incluyendo algunos cereales, secretan proteínas al apoplasto que se adsorben a la superficie de los cristales de hielo extracelular e inhiben o modifican su crecimiento. Estas proteínas son denominadas AFP (del inglés *antifreezing proteins*) (Griffith y Yaish, 2004). En centeno se ha observado que

las AFPs son similares a las PRs de plantas pero se encuentran en complejos oligoméricos (Yu y Griffith, 1999). Asimismo, se confirmó por secuenciación del extremo N-terminal, que algunas de las proteínas abundantes que tuvieron actividad anticongelante *in vitro* eran quitinasas, β-1,3-glucanasas, y proteínas tipo taumatina (Hon, 1994).

Yu et al., (2001) investigaron el papel del SA y del etileno en la inducción de PRs con o sin actividad anticongelante. Mientras que el SA indujo PRs que no tenían actividad anticongelante, el etileno incrementó la acumulación de otras que sí la tenían. Además, sólo algunas isoformas mostraron actividad antifúngica y otras presentaron funciones adicionales como actividad anticongelante (Sela Buurlage et al., 1993; Yeh et al., 2000). Yaish et al. (2006) caracterizaron β-1,3-glucanasas y β-1,3;1,4-glucanasas procedentes de centeno y observaron que eran capaces de inhibir la formación de cristales de hielo, potencialmente fatales para la planta, además de tener actividad enzimática con un papel potencial en resistir la infección por patógenos psicrófilos.

En variedades de *Cynodon* sp. tolerantes a bajas temperaturas se observó un incremento en los niveles de expresión de un gen de quitinasa de clase II (de los Reyes et al., 2001). Además, se ha caracterizado una proteína tipo osmotina (PR-5) aislada de dulcámara (*Solanum dulcamara*) con función crioprotectora (Newton y Duman, 2000). También se ha descrito el papel crioprotector de las PRs en proteínas de la familia PR-2 de tabaco (Hincha et al., 1997), y PR-5 de cacahuete (Dave y Mitra 1998).

Además de las funciones de las quitinasas y β-1,3-glucanasas citadas hasta el momento (como antifúngicas, crioprotectoras y/o anticongelantes) importantes en el mecanismo de defensa de las plantas; la trascendencia de las quitinasas de clase I en los últimos años se debe a que diversos estudios han demostrado que estas proteínas son los principales panalergenos asociados con el síndrome designado látex-fruta (Poley y Slater, 2000). Además de en el látex, se han identificado varias proteínas asociadas a diferentes

PRs en distintos frutos (Receveur-Bréchot et al., 2006). Varios estudios han identificado las quitinasas de clase I como importantes componentes de unión a IgE en suero de pacientes con síndrome de látex-fruta en plátano (Sanchez-Monge et al., 1999), aguacate (Sowka et al., 1998) y castaña (Díaz-Perales et al., 1998). En 2002, O´Riordain et al. clonaron y caracterizaron un alergeno de *Hevea brasilensis* que presentó homología con una quitinsa de clase I. La proteína recombinante mostró tener actividad biológica e inmunológica. También se han identificado β-1,3-glucanasas (PR-2) entre los mayores alergenos responsables de la alergia al látex.

En uva, Robinson et al. (1997) clonaron y caracterizaron dos cDNAs que codifican quitinasa y observaron que la actividad de la misma aumentaba marcadamente al comienzo de la maduración de las bayas y continuaba aumentando durante la fase de acumulación de azúcares. Derckel et al. (1998) caracterizaron isoformas de quitinasa y β-1,3-glucanasas en diferentes estados de maduración de las bayas pero no detectaron actividad glucanasa en ninguno de ellos. Además, añadiendo SA a los racimos infectados por hongo, se observó que sólo se potenciaba la actividad quitinasa de los granos infectados. Busam et al. (1997) observaron que la expresión del gen de una quitinasa de clase III podría servir como un marcador de la SAR en hojas de vid. Por otra parte, existen estudios en granos de uva infectada por *B. cinerea* donde no se han detectado acúmulos de tránscritos de quitinasas de clase I en ninguna etapa del desarrollo (Robert et al., 2002), lo que podría indicar que su regulación estuviera relacionada con la temperatura de almacenamiento de la uva.

Con el fin de profundizar en los mecanismos moleculares implicados en la repuesta de uva de mesa a las bajas temperaturas y al tratamiento con altos niveles de CO_2, se estudiaran los niveles de expresión, de actividad, así como las posibles funciones *in vitro* de una quitinasa y de una β-1,3-glucanasa de clase I a lo largo de la conservación de los frutos. Para ello se realizará el aislamiento de genes que codifican una quitinasa y β-1,3-

glucanasa de clase I, así como su expresión heteróloga en *E.coli* para llevar a cabo la caracterización de las proteínas recombinantes.

ARTÍCULO 5

Expression of class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO_2 pretreatment.

Irene Romero, María T. Sanchez-Ballesta, Roberto Maldonado, María I. Escribano, Carmen Merodio.

Postharvest Biology and Technology 41 (2006) 9-15

RESUMEN

El efecto del pretratamiento con 20% CO_2 más 20% O_2 durante 3 días fue estudiado con el fin de estudiar su efectividad en el control del ataque por hongo crecido de forma natural durante la manipulación poscosecha y su posible inducción de genes específicos de PRs en uvas de mesa. CDNAs de tamaño completo que codificaban una quitinasa de clase I (*VcCHIT1b*) y una β-1,3-glucanasa (*VcGNS1*) fueron aislados de uvas de mesa (*Vitis vinifera* L. cv. 'Cardinal'). Nuestros resultados indicaron que este pequeño tratamiento con alto CO_2 tuvo un efecto residual y redujo significativamente la incidencia de daño por hongo en uvas de mesa durante el almacenamiento a bajas temperaturas y después de su transferencia a 20ºC. Nuestros resultados indican que durante el almacenamiento a bajas temperaturas el patrón de expresión fue diferente entre los genes de las dos PR estudiadas. Así, mientras la abundancia del transcrito *VcGNS1* aumentó fuertemente al comienzo del almacenamiento a 0ºC, el aumento de los niveles de mRNA de *VcCHIT1b* fue paralelo al cambio por ataque fúngico. El pretratamiento con altas concentraciones de CO_2 restringió el aumento de la expresión génica de *VcGNS1* y retrasó la acumulación del transcrito *VcCHIT1b* en comparación con uvas no tratadas. Después del paso a 20ºC después de 33 días almacenadas en frío, cuando fue observado los niveles máximos de ataque por hongo, hubo un gran aumento en la acumulación de mRNA de *VcCHIT1b* tanto en uvas tratadas como no tratadas, el cual fue mayor en las no tratadas. Nuestros resultados señalan que la expresión de los genes de quitinasa de clase I y de β-1,3-glucanasa no está aumentada en uvas tratadas con CO_2, el cual controla el ataque fúngico. Estos resultados sugieren, entonces, que la eficacia del pretratamiento con altas concentraciones de CO_2 en reducir el ataque por hongo no está mediada por la inducción de los genes de las PRs anteriormente mencionadas.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Postharvest Biology and Technology 41 (2006) 9–15
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

Expression of class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO_2 pretreatment

Irene Romero [1], María T. Sanchez-Ballesta [1], Roberto Maldonado, María I. Escribano, Carmen Merodio *

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frío, IF-CSIC, José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain

Received 8 November 2005; accepted 2 March 2006

Abstract

The effect of pretreatment with 20% CO_2 plus 20% O_2 for 3 days was studied with regard to its effectiveness on natural postharvest decay control and its possible induction of specific PR genes in table grapes. Full-length cDNAs encoding a class I chitinase (*Vcchit1b*) and β-1,3-glucanase (*Vcgns1*) were isolated from table grapes (*Vitis vinifera* L. cv. 'Cardinal'). Our results indicate that this short-term high CO_2 treatment had a residual effect and significantly reduced decay incidence of table grapes during low temperature storage and upon transfer to 20 °C. Our results indicate that during low temperature storage the expression pattern differed between the two tested PR genes. So, while the abundance of *Vcgns1* transcript increased sharply at the beginning of storage at 0 °C, the increase in *Vcchit1b* mRNA levels was paralleled by the change in total decay. High CO_2 pretreatment restrained the up-regulation of *Vcgns1* gene expression and delayed the accumulation of *Vcchit1b* transcript as compared with non-treated grapes. Upon transfer to 20 °C after 33 days of cold storage, when attainment of maximum total decay was observed, there was a sharp increase in the accumulation of *Vcchit1b* mRNA in both treated and non-treated grapes, which was higher in the non-treated ones. Our results point out that the expression of class I chitinase and β-1,3-glucanase genes is not enhanced in CO_2-treated grapes which control total fungal decay. These results suggest, then, that the efficacy of high CO_2 pretreatment in reducing total fungal decay is not mediated by induction of the above-mentioned PR genes.

Keywords: Table grapes; Postharvest technology; Carbon dioxide; *Botrytis cinerea*; Pathogenesis-related proteins; Gene expression

1. Introduction

Postharvest deterioration of table grapes normally results from fungal decay, largely caused by *Botrytis cinerea*, and desiccation of stems and pedicels, which limits prolonged storage of grapes at low temperature. Control against *Botrytis* disease during storage and transportation of grapes is currently achieved by application of fungicides (Luvisi et al., 1992). However, fungicides and chemical treatments may cause damage to grape berries if used excessively, and some consumers develop allergic reactions. Moreover, frequent prophylactic use of fungicides may lead to multiple resistance in the pathogen population (Raposo et al., 1996; Alfonso et al., 2000). Some alternative strategies have been tried in the place of fungicides (Yahia et al., 1983; Crisosto et al., 2002; Lydakis and Aked, 2003; Retamales et al., 2003). However, to evaluate the efficacy of postharvest treatments for control of decay, we need to know whether these treatments have a direct effect on the fruit's defense responses.

Plant resistance is correlated with the activation of several defense mechanisms including the transcriptional activation of numerous defense-related genes, deposition of mechanical barriers, accumulation of phytoalexins and synthesis of specific pathogenesis-related (PR) proteins. Among PR pro-

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: merodio@if.csic.es (C. Merodio).
[1] These authors contributed equally to this work.

0925-5214/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.postharvbio.2006.03.001

teins, the most fully characterized enzymes are chitinases and β-1,3-glucanases which hydrolyze polymers of fungal cell walls, and it has therefore been suggested that both enzymes are involved in plant defense mechanisms against fungal infection (Boller, 1985; Collinge et al., 1993). Chitinases and β-1,3-glucanases, generally encoded by multigenic families, have been classified by sequence similarity into six and four families, respectively. Also, various studies have demonstrated that transgenic plants overexpressing chitinase and β-1,3-glucanase genes show enhanced resistance to fungal infection (Zhu et al., 1994; Grison et al., 1996).

Multiple isoforms of chitinase and β-1,3-glucanase have been reported in grapes (Busam et al., 1997; Robinson et al., 1997), but the available data concerning their expression in grape berries are limited. Increased levels of chitinases and glucanase mRNAs, or their activities, have been reported in grapevine leaves infected by *Botrytis cinerea* (Derckel et al., 1996; Renault et al., 1996). Busam et al. (1997) reported that the expression of a class III chitinase gene might serve as a marker for systemic acquired resistance in grapevine leaves. In stored tropical fruit, coordinate accumulation of PR proteins such as chitinase-like protein and β-1,3-glucanase has been reported in fruit able to withstand chilling temperature storage (Merodio et al., 1998). However, there are no studies showing changes in the transcript accumulation of defense-related genes in response to high CO_2 levels.

The purpose of this study was to investigate whether the effect of 20% CO_2 on natural postharvest decay control of table grapes directly affects the expression of specific PR genes. The effect of pretreatment with 20% CO_2 on chitinase activity and expression of cDNAs encoding a class I chitinase (*Vcchit1b*) and β-1,3-glucanase (*Vcgns1*) was analyzed. Our results indicate that the tested PR genes are not induced in CO_2-treated grapes that are able to control fungal attack. High CO_2 pretreatment may have an indirect effect on the expression of the class I chitinase gene, and it has a clear effect on avoiding class I β-1,3-glucanase gene induction.

2. Materials and methods

2.1. Plant material

Table grapes (*Vitis vinifera* L. cv. Cardinal) were harvested at random in Camas (Sevilla, Spain) in July. Early-harvest mature berries were used in this work (12.7% total soluble solids; 0.81% tartaric acid). Immediately after harvesting, field-packaged bunches were transported to the laboratory, where fruit were forced-air-cooled for 14 h at −1 °C. After cooling, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 °C and 95% relative humidity (RH) in two sealed neoprene containers of 1 m^3 capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container. One lot was stored under normal atmosphere for 33 days (non-treated fruit) and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. This CO_2 concentration was maintained during the pretreatment experiment and it was measured daily using an automated system gas chromatograph equipped with a thermal conductivity detector and Poraplot Q column (Varian Chrompack CP20033P). After 3 days, CO_2-treated grapes were transferred to air under the same conditions as the non-treated fruit until the end of the storage period. After 12 and 33 days CO_2-treated and non-treated grapes were transferred to ventilated storage containers for 2 days at 20 °C and 95% RH, to simulate shelf-life during marketing. Ten clusters were sampled periodically during low temperature storage and at the end of their shelf-life. Berries obtained from five clusters (approximately 300 g each cluster) were peeled and the skin was frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis.

2.2. Evaluation of storage decay

Storage decay was evaluated on the basis of the total decay after removing and weighing the healthy berries. The weight of the decayed berry was calculated by subtracting healthy berries from the total cluster weight. Thus, total decay was expressed as a percentage of decayed berries with respect to the original cluster weight.

2.3. cDNA cloning

Total RNA was isolated from 4 g of frozen berry skin tissues according to Salzman et al. (1999). RT-PCR and 5′-3′ RACE were used in cloning the full-length cDNA clones of class I chitinase and β-1,3-glucanase. Partial cDNA clones of class I chitinase and β-1,3-glucanase were obtained by RT-PCR. cDNA synthesis was performed with 10 μg of total RNA from the skin tissues of non-treated and CO_2-treated grapes stored at 0 °C. The reaction was carried out in the presence of 500 ng of oligo-dT with 100 units of Reverse Transcriptase (Ecogen). Chitinase and β-1,3-glucanase gene DNA fragments were obtained by PCR amplification using the cDNA as template and degenerate oligonucleotide primers complementary to conserved peptide regions of the class I chitinase and β-1,3-glucanase. A 550-bp fragment of class I chitinase was amplified by combining the sense primer 5′-TGC/T TGC AGC AAG/A TTC/T GGC/T TG/TG/C TG-3′, corresponding to the peptide motif CCS(K/Q)FG(F/W)C of the chitin-binding domain, and the antisense primer 5′-A/C/T/GGA C/TTG A/C/T/GGG A/C/T/GGT CAT CCA GAA CCA-3′ derived from the peptide WFWMT(A/P)QS. A 850-bp fragment of class I β-1,3-glucanase was amplified using a sense primer 5′-TA/C/T/GG GTG TA/C/T/GT GC/TT ATG GAA TGC T-3′ derived from the conserved peptide VGVCYGML, and the antisense primer 5′-CTC A/GTC AAA CAT G/AGC AAA A/T/C/GAG/T/A G/A-3′ corresponding to the peptide (L/I)FAMFDE. Both PCR-fragments were cloned into pGEMT (Promega) and confirmed by sequencing. The sequences were used to select

oligonucleotide primers for performing both 5′- and 3′-rapid amplification of cDNA ends (RACE) to obtain the full-length. The 5′/3′ RACE kit (Roche) was applied according to the manufacturer's instructions using total RNA isolated to synthesize the cDNA mentioned above. The chitinase and β-1,3-glucanase 5′ and 3′ PCR-RACE products were subcloned and sequenced. The sequences of these PCR-RACE products and the chitinase 550-bp and β-1,3-glucanase 850-bp internal PCR products yielded overlapping PCR products extending to the 5′ and 3′ ends of the cDNA.

2.4. Northern-blot hybridization

Samples of denatured total RNA (10 μg) from the skin were fractionated and blotted as described in Sanchez-Ballesta et al. (2000). Equal loading was confirmed by ethidium bromide staining and by membrane staining with methylene blue. As probes we used the full-length cDNAs *Vcchit1b* and *Vcgns1* random-primer labeled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 °C in 7% sodium dodecyl sulfate, 0.33 M phospate buffer, pH 7.2 and 1 mM EDTA, then washed twice in 2× SSC, 0.1% SDS at room temperature and twice in 0.1× SSC, 0.1% SDS at 65 °C and exposed to Kodak X-Omat SX film at −80 °C. Autoradiographs were digitally scanned and band densities quantified by 20 image densitometry using Scion Image Software (Scion Corporation, Frederick, MD). The 100% was assigned to the maximum optical density value achieved in each northern and the rest of optical densities were normalized to the maximum value and expressed as percentage of relative accumulation (RA).

2.5. Chitinase extraction and activity

Protein was extracted by homogenizing ground frozen berry skin tissues (250 mg fresh weight) at 4 °C in 5 mL of 100 mM sodium acetate buffer, pH 5.0 and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 27,000 × *g* for 30 min at 4 °C and the clarified supernatant was recovered. Chitinase activity was assayed using a commercial blue enzyme substrate, CM-chitin-RBV solution (Loewe), based on the precipitability of a non-degraded, highly polymerized substrate when acid is added. Chitinase activity was determined utilizing different dilutions from the crude extract until a linear range of activity versus substrate was established. Enzyme activity was assayed by incubating a standard reaction mixture containing 70 μL of diluted crude enzyme extract (14 μL of original crude extract), 200 μL of aqueous CM-chitin-RBV (2 mg mL^{-1}) and 100 mM sodium acetate buffer, pH 5.0 to yield a final reaction volume of 0.8 mL, for 15 min at 37 °C. The reaction was stopped by adding 200 μL of 2N HCl and cooling on ice for 10 min. The mixture was then centrifuged at 10,000 × *g* for 5 min to precipitate the non-degraded substrate. The supernatant containing degraded polymers was diluted (1:1, v/v) with nanopure water and absorbance was measured at 550 nm against a blank reaction (incubation mixture with HCl-treated crude extract). Specific enzyme activity was defined as "absorbance at 550 nm h^{-1} mg^{-1} of protein". Protein concentration was measured by the Bradford (1976) method using a protein-dye reagent (Bio-Rad) and BSA as a standard.

2.6. Statistical analyses

Data from at least three replicates per sample were subjected to analysis of variance (ANOVA) (Stargraphics Program, STSC, Rockville, MD). Multiple variance analysis was employed to determine the significance of the data at $P \leq 0.05$. The results presented here are representative of data from two separate experiments performed in succession.

3. Results

3.1. Isolation and sequence analysis of Vcchit1b and Vvgns1 genes

The full lengths of the *Vcchit1b* (GeneBank Accession no. DQ267094) and *Vcgns1* (GeneBank Accession no. DQ267748) cDNAs were isolated using RT-PCR and 5′-3′ RACE strategies. The *Vcchit1b* cDNA consisted of 1133 bp with an open reading frame of 942 bp and encoded a polypeptide of 314 amino acids. The amino acid sequence of VcCHIT1b shared 100% identity with the class I chitinases VCHIT1b (Busam et al., 1997) and VvCHIT1a (Robert et al., 2002), previously isolated from 'Ugni Blanc' and 'Pinot Noir' cultivars, respectively. In addition, sequence domains typical of class I chitinase, such as a putative signal peptide of 20 amino acids followed by a cysteine-rich chitin-binding domain, were found in the N-terminal sequence. A hinge domain of only three amino acid residues separated the cysteine-rich domain from the catalytic domain. Also, the VcCHIT1b protein contained a C-terminal extension of at least seven amino acids, suggesting a vacuolar localization. The mature protein of 286 amino acids presented a calculated p*I* of 8.44 and a calculated molecular mass of 31.27 kDa.

The full-length cDNA sequence of the *Vcgns1* consisted of 1316 bp and contained an open reading frame of 1080 bp encoding a protein of 360 amino acid residues. The amino acid sequence of VcGNS1 shared 69.1 and 74.5% identity with the basic class I β-1,3-glucanases of *Hevea brasiliensis* and *Nicotiana plumbaginifolia* (*gns1*) (Chye and Cheung, 1995; Castresana et al., 1990), respectively. Comparison with other β-1,3-glucanases in the databases revealed similarities of only 40–55%. The deduced amino acid sequence of VcGNS1 contains both N- and C-terminal signal peptide sequences of 22 and 21 amino acids, respectively, present in the class I β-1,3-glucanase proteins and required for targeting the protein to the vacuole (Meins et al., 1992). It was assumed that N- and C-terminal processing occurs in a manner similar to that reported for class I β-1,3-glucanase from

tobacco (Shinshi et al., 1988), a mature protein of 317 amino acids with a calculated p*I* of 9.68 and a molecular mass of 34.6 kDa.

3.2. Effect of high CO_2 levels on total fungal decay during postharvest storage at low temperature

In non-treated grapes, total decay increased progressively during postharvest storage at 0 °C, reaching levels of about 25% after 33 days. The beneficial effect of high CO_2 levels on gray mold incidence was demonstrated since the first diseased berries (1.7% of the total decay) were detected only after 22 days of storage (Fig. 1), while in non-treated fruit the onset of the increase was observed after 12 days. The levels attained at the end of storage (33 days) were significantly lower in CO_2-treated fruit than those observed in non-treated fruit.

3.3. Effect of high CO_2 levels on class I chitinase and β-1,3-glucanase mRNA levels and chitinase activity in table grapes during postharvest storage at low temperature

In the skin of non-treated grapes, low-temperature storage drastically increased levels of the *Vcgns1* mRNA; these remained stable throughout storage, and a decrease in abundance was observed only after 33 days. However, in CO_2-treated grapes although accumulation of the *Vcgns1* transcript also increased rapidly at 0 °C, this increase was smaller than in non-treated grapes (Fig. 2A). Storage at 0 °C also induced a slow but progressive accumulation of the *Vcchit1b* transcript in the grape skin, reaching the maximum by day 28 and decreasing slightly thereafter (Fig. 2A). However, the CO_2 treatment delayed accumulation of the class I chitinase mRNA levels, increasing after 22 days and remaining stable until the end of storage (Fig. 2A). With regard to chitinase activity (Fig. 2B), it is interesting to note that although activity did not increase progressively during storage at 0 °C as occurred with the *Vcchit1b* mRNA levels, the transient peaks in the activity were observed at the time of the increases in accumulation of the transcript (12 and 28 days) (Fig. 2A). By comparison, in CO_2-treated grapes the transient increase in the chitinase activity was observed after 22 days of storage, when an increase in the accumulation of *Vcchit1b* transcript was observed.

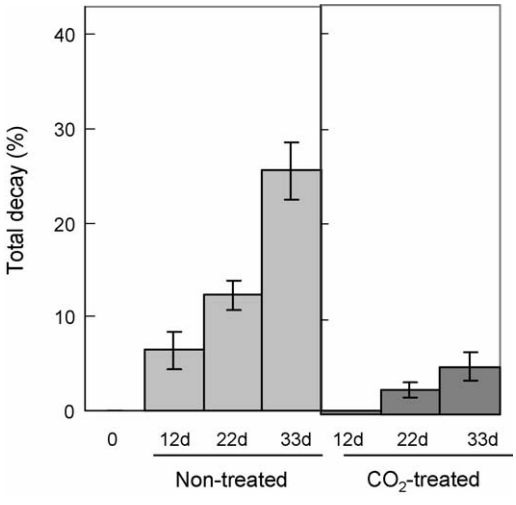


Fig. 1. Total decay (%) in non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 °C for 12, 22 and 33 days. Values are the means of three replicate samples ± S.E.

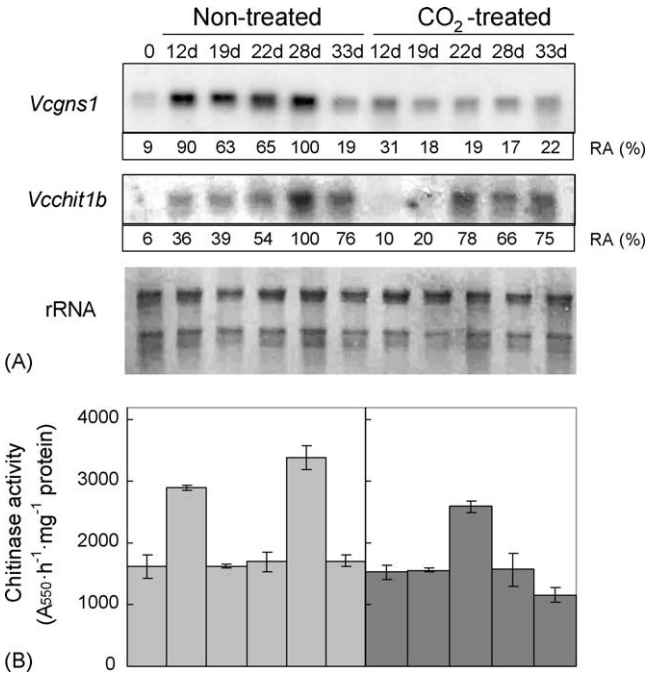


Fig. 2. Effect of high CO_2 levels on *Vcgns1* and *Vcchit1b* mRNA accumulation in the skin of 'Cardinal' table grapes stored at 0 °C (A). Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *Vcchit1b* and *Vcgns1* probes. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical densities values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. (B) Chitinase activity patterns in the skin of non-treated and CO_2-treated 'Cardinal' table grapes during storage at 0 °C. Enzyme activity was expressed as absorbance at 550 nm h^{-1} mg^{-1} of protein. Data are averages of two separate experiments ($n = 6$) and S.E. are shown by vertical bars.

3.4. Changes in class I chitinase gene expression, chitinase activity and total decay during the transfer of CO_2-treated and non-treated grapes to 20 °C

After 12 and 33 days of storage at 0 °C, changes in *Vcchit1b* gene expression (Fig. 3A), chitinase activity (Fig. 3B) and total fungal decay (Fig. 3C) in CO_2-treated and non-treated fruit transferred to 20 °C during 2 days, were analyzed. Upon transfer after 12 days of storage, the levels of *Vcchit1b* transcript and chitinase activity in non-treated grapes decreased slightly. In CO_2-treated fruit a slight increase was observed in both *Vcchit1b* gene expression and chitinase activity. The transfer to 20 °C after 33 days of cold storage drastically increased the levels of *Vcchit1b* transcript

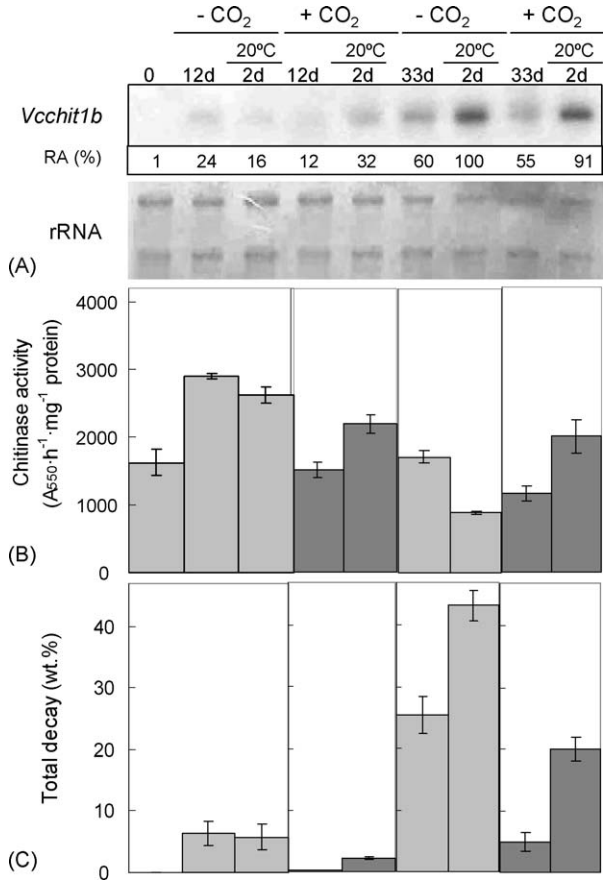


Fig. 3. The pattern of change of mRNA levels (A), chitinase activity (B) and total decay (C) in the skin tissues of non-treated and CO_2-treated 'Cardinal' table grapes stored 12 and 33 days at 0 °C and transferred to 20 °C for 2 days. (A) Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *Vcchit1b* probe. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical densities values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. (B) Enzyme activity was expressed as absorbance at 550 nm h^{-1} mg^{-1} of protein. Data are averages of two separate experiments ($n = 6$) and S.E. are shown by vertical bars. (C) Total decay (%) values are the means of three replicate samples ± S.E.

in skin tissues of both non-treated and CO_2-treated grapes, but this increase was even higher in non-treated ones. This sharp increase in *Vcchit1b* transcript did not correlate with the chitinase activity. However, it was consistent with the total decay increases observed in both series of samples.

4. Discussion

Advances in postharvest handling of table grapes, including the development of treatments for controlling storage decay while avoiding the use of chemicals, have been the focus of interest in recent years (Palou et al., 2002; Chervin et al., 2005). Most of the research has focused on the effectiveness of different postharvest treatments to counteract disease development in table grapes inoculated with *B. cinerea*. The use of controlled atmosphere (CA) packaging has been proposed as a suitable treatment to replace SO_2 fumigation as a means of controlling grape decay (Yahia et al., 1983; Crisosto et al., 2002; Artés-Hernéndez et al., 2003). However, in the case of high CO_2 concentrations, applied as a pretreatment for short periods of time, there is little information about its effectiveness in retarding the appearance of symptoms caused by fungal attack and its possible mode of action. Therefore, with a view to assess how effectively high CO_2 concentrations are able to activate specific defense mechanisms against fungal attack, we focused on an analysis of PR gene expression in CO_2-treated and non-treated grapes. Class I chitinase (*Vcchit1b*) and β-1,3-glucanase (*Vcgns1*) cDNAs were isolated from grapes of the 'Cardinal' cultivar of *V. vinifera*. The *Vitis* chitinase and β-1,3-glucanase analyzed here were structurally very similar to the corresponding proteins from *Vitis* or other species. The VcCHIT1b protein is composed of three domains present in the class I chitinase: a Cys rich chitin-binding domain, a Pro rich hinge region and a highly conserved catalytic domain. Moreover, the class I chitinase appeared to be very well preserved in *Vitis*, as the deduced VcCHIT1b protein was identical to the vacuolar class I chitinase from leaves of 'Ugni Blanc' (Robert et al., 2002) and cell cultures of 'Pinot Noir' (Busam et al., 1997). The predicted amino acid sequence of VcGNS1 contains a preserved tryptophan residue, which is implicated in the interaction with the glucan substrate (Ori et al., 1990), and a preserved glutamate, which has been shown to act as a nucleophile in the catalytic mechanism (Varghese et al., 1994). The deduced amino acid sequence of VcGNS1 contains both N- and C-terminal signal peptides, class I β-1,3-glucanases are synthesized as preproteins and the N- and C-terminal extensions are cleaved during or after transport of the protein to the vacuole (Shinshi et al., 1988).

While expression of chitinase and β-1,3-glucanase genes has been extensively studied in plants subjected to specific stress conditions, there is no information in response to high CO_2 levels during postharvest storage applied against fungal infection. Our results indicate that in table grapes cv. Cardinal, the enhanced expression of a cDNA encoding β-1,3-glucanase (*Vcgns1*) (Fig. 2A) observed in non-treated grapes during low temperature storage was constrained by pretreatment with high CO_2 levels, using as a probe the full-length cDNA. Moreover, in these conditions we cannot find any relation between this gene and fungal infection. The marked accumulation of *Vcgns1* mRNA in non-treated grapes at the beginning of storage at 0 °C may indicate that factors other than fungal infection are involved in its induction. In this connection, mRNA accumulation of a class II and class III β-1,3-glucanase from tomato and mandarin, respectively, increased during chilling temperature storage (Ding et al., 2002; Sanchez-Ballesta et al., 2006). Although the effect of low temperature on the expression of β-1,3-glucanase requires further study, high CO_2 levels appears to be a clear effect in avoiding the induction of class I β-1,3-glucanase

genes. The accumulation of *Vcchit1b* transcript (Fig. 2A) during low temperature storage was paralleled by the change in total decay (Fig. 1), using as a probe the full-length cDNA. In this work we observed that total decay increased gradually in non-treated grapes stored at 0 °C. In CO_2-treated grapes, on the other hand, total decay had not increased after 22 days of storage, and class I chitinase gene expression was delayed. With regard to chitinase activity, during low temperature storage the sharp transient increases in total activity matched the increase in *Vcchit1b* transcript levels. Moreover, a differential increase in chitinase activity was observed when the increase in *Vcchit1b* transcript levels was above 30% in both non-treated and CO_2-treated grapes. Upon transfer to 20 °C after 33 days of storage at 0 °C, there was a sharp increase in the accumulation of *Vcchit1b* mRNA in both the treated and non-treated grapes, although it was smaller in the CO_2-treated fruit. With regard to chitinase activity, the transfer to 20 °C at the end of the storage period in non-treated fruit was associated with a decrease in the levels of chitinase activity, although the expression of class I chitinase was sharply enhanced. At this time, chitinase activity in the skin tissues of non-treated grapes decreased significantly, reaching values 45% lower that those quantified in freshly harvested grapes and 55% lower than in CO_2-treated grapes. In CO_2-treated grapes, contrary to what was observed in non-treated grapes, the chitinase activity increased by about 73% with respect to the values prior to transfer. These results are consistent with the change in total decay observed in both series of samples. Effective control against *B. cinerea* and *Penicillium digitatum* has been reported by means of endochitinase combined in mixtures with other antifungal compounds (Ali et al., 2003). During transfer to 20 °C, the lack of correlation between *Vcchit1b* transcript levels and chitinase activity, mostly in non-treated grapes, could be explained in terms of the kinetic and regulatory properties of chitinase enzyme. Many factors, including different post-translational modifications, could be involved in the modulation of chitinase activity. Several isoforms of chitinase have been identified, and it is generally considered that these isoforms represent the products of different members of the chitinase gene family. Additional research is required to study possible mechanisms of regulation of chitinase activity and may provide some useful insights to help restrain fungal infection.

In this work a close link has been established between high CO_2 pretreatment and control of fungal decay. Our results indicate that the expression of class I chitinase and β-1,3-glucanase genes is not enhanced in CO_2-treated grapes that are able to control fungal decay. However, in CO_2-treated grapes, contrary to what was observed in non-treated grapes, there was an upward trend in chitinase activity at the time of maximum growth of fungal decay at 20 °C. We suggest that a mechanism other than induction of the tested PR genes renders grape berries less susceptible to natural postharvest fungal infection. Elsewhere, we reported that the application of 20% CO_2 for short periods of time was effective in reducing the senescence-like responses in peel tissues of fruit (Escribano et al., 1997) and controlling specific processes associated with low temperature storage (Maldonado et al., 2002). Further analysis is needed to ascertain the mechanism whereby CO_2 treatment can influence the tolerance of table grape to fungal attack before the appearance of the first symptoms and may provide some useful insights to help restrain fungal infection during prolonged low temperature storage.

Acknowledgements

This work was supported by research grants (AGL2002-02308/ALI and AGL2005-04502/ALI) from CICYT (Spain). M.T.S.-B. and R.M. were supported by a postdoctoral contract from Comunidad de Madrid and by a fellowship from MCyT (Spain), respectively. The authors are grateful to Frutaria SAT for providing tables grapes.

References

Alfonso, C., Raposo, R., Melgarejo, P., 2000. Genetic diversity in *Botrytis cinera* populations on vegetable crops in greenhouses in south-eastern Spain. Plant Pathol. 49, 243–251.

Ali, G.S., Harman, G.E., Reisch, B.I., 2003. The interaction of endochitinase, a synthetic peptide and resveratrol in controlling fungi in vitro. Eur. J. Plant Pathol. 109, 639–644.

Artés-Hernéndez, F., Artes, F., Tomás-Barberán, F.A., 2003. Quality and enhancement of bioactive phenolics in cv. Napoleon table grapes exposed to different postharvest gaseous treatments. J. Agric. Food Chem. 51, 5290–5295.

Boller, T., 1985. Induction of hydrolases as a defense reaction against pathogens. In: Key, J.L., Kosuge, T. (Eds.), Cellular and Molecular Biology of Plant Stress. UCLA Symp. Mol. Cells Biol. NS, vol. 22, pp. 247–262.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Busam, G., Kassemeyer, H., Matern, U., 1997. Differential expression of chitinases in *Vitis vinifera* L. Responding to systemic acquired resistance activators or fungal challenge. Plant Physiol. 115, 1029–1038.

Castresana, C., de Carvalho, F., Gheysen, G., Habets, M., Inze, D., Van Montagu, M., 1990. Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* beta-1,3-glucanase gene. Plant Cell 2, 1131–1143.

Chervin, C., Westercamp, P., Monteils, G., 2005. Ethanol vapours limit *Botrytis* development over the postharvest life of table grapes. Postharvest Biol. Technol. 36, 319–322.

Chye, M.L., Cheung, K.Y., 1995. Beta-1,3-glucanase is highly-expressed in laticifers of Hevea brasiliensis. Plant Mol. Biol. 29, 397–402.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1993. Plant chitinases. Plant J. 3, 31–40.

Crisosto, C.H., Garner, D., Crisosto, G., 2002. Carbon dioxide-enriched atmospheres during cold storage limit losses from Botrytis but accelerate rachis browning of 'Redglobe' table grapes. Postharvest Biol. Technol. 26, 181–189.

Derckel, J.P., Legendre, L., Audran, J., Haye, B., Lambert, B., 1996. Chitinases of the grapevine (*Vitis vinifera* L): five isoforms induced in leaves by salicylic acid are constitutively expressed in other tissues. Plant Sci. 119, 31–37.

Ding, C.K., Wang, C.Y., Gross, K.C., Smith, D.L., 2002. Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes

and increase resistance to chilling injury in tomato fruit. Planta 214, 895–901.

Escribano, M.I., Del Cura, B., Muñoz, T., Merodio, C., 1997. The effect of high carbon dioxide at low temperature on ribulose 1,5-biphosphate carboxylase and polygalacturonase protein levels in cherimoya fruit. J. Am. Soc. Hort. Sci. 122, 258–262.

Grison, R., Grezesbesset, B., Scheneider, M., Lucante, N., Olsen, L., Legauay, J.J., Toppan, A., 1996. Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nat. Biotechnol. 14, 643–646.

Luvisi, D.A., Shorey, H., Smilanick, J., Thompson, J., Gump, B., Knutson, J., 1992. Sulfur Dioxide Fumigation of Table Grapes. University of California, DANR, Bulletin 1932.

Lydakis, D., Aked, J., 2003. Vapour heat treatment of Sultanina table grapes. I: control of *Botrytis cinerea*. Postharvest Biol. Technol. 27, 109–116.

Maldonado, R., Molina-García, A.D., Sanchez-Ballesta, M.T., Escribano, M.I., Merodio, C., 2002. High CO_2 atmosphere modulating the phenolic response associated with cell adhesion and hardening of *Annona cherimola* fruit stored at chilling temperature. J. Agric. Food Chem. 50, 7564–7569.

Meins Jr., F., Neuhaus, J.M., Ryals, J., 1992. The primary structure of plant pathogenesis-related glucano-hydrolases and their genes. In: Boller, T., Meins, F. (Eds.), Genes Involved in Plant Defence. Springer-Verlag, Wien, pp. 245–282.

Merodio, C., Muñoz, M.T., Del Cura, B., Buitrago, D., Escribano, M.I., 1998. Effect of high CO_2 level of γ-aminobutyric acid, total polyamines and some pathogenesis-related proteins in cherimoya fruit stored at low temperature. J. Exp. Bot. 49, 1339–1347.

Ori, N., Sessa, G., Lotan, T., Himmelboch, S., Fluhr, R., 1990. A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass. EMBO J. 9, 3249–3436.

Palou, L., Crisosto, C.H., Smilanick, J.L., Adaskaveg, J.E., Zoffoli, J.J.P., 2002. Effects of continuous 0.3 ppm ozone exposure on decay development and physiological responses of peaches and table grapes in cold storage. Postharvest Biol. Technol. 24, 30–48.

Raposo, R., Delcan, J., Gomez, V., Melgarejo, P., 1996. Distribution and fitness of isolates of *Botrytis cinerea* with multiple fungicide resistance in Spanish greenhouses. Plant Pathol. 45, 497–505.

Renault, A., Deloire, A., Bierne, J., 1996. Pathogenesis-related proteins in the grapevine induced by salicylic acid and *Botrytis cinerea*. Vitis 35, 49–52.

Retamales, J., Defilippi, B.G., Arias, M., Castillo, P., Manríquez, D., 2003. High-CO_2 controlled atmospheres reduce decay incidence in Thompson Seedless and Red Globe table grapes. Postharvest Biol. Technol. 29, 177–182.

Robert, N., Roche, K., Lebeau, Y., Breda, C., Boulay, M., Esnault, R., Buffard, D., 2002. Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. Plant Sci. 162, 389–400.

Robinson, S.P., Jacobs, A.K., Dry, I.B., 1997. A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiol. 114, 771–778.

Salzman, R.A., Fujita, T., Zhu-Salzman, K., Hasegawa, P.M., Bressan, R.A., 1999. An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant. Mol. Biol. 17, 11–17.

Sanchez-Ballesta, M.T., Lafuente, M.T., Zacarias, L., Granell, A., 2000. Involvement of phenylalanine ammonia-lyase in the response of Fortune mandarin fruits to cold temperature. Physiol. Plant. 108, 382–389.

Sanchez-Ballesta, M.T., Gosalbes, M.J., Rodrigo, M.J., Granell, A., Zacarias, L., Lafuente, M.T., 2006. Characterization of a β-1,3-glucanase from citrus fruit as related to chilling-induced injury and ethylene production. Postharvest Biol. Technol., in press.

Shinshi, H., Wenzler, H., Neuhaus, J.M., Felix, G., Hofsteenge, J., Meins Jr., F., 1988. Evidence for N- and C-terminal processing of a plant defense-related enzyme: primary structure of tobacco prepo-β-1,3-glucanase. Proc. Natl. Acad. Sci. U.S.A. 85, 5541–5545.

Varghese, J.N., Garret, T.P.J., Colman, P.M., Chen, L., Hoj, P.B., 1994. Three-dimensional structures of two β-glucan endohydrolases with distinct substrate specificities. Proc. Natl. Acad. Sci. U.S.A. 91, 2785–2789.

Yahia, E.M., Nelson, K.E., Kader, A.A., 1983. Postharvest quality and storage life of grapes as influenced by adding carbon monoxide to air or controlled atmospheres. J. Am. Sci. Hort. Sci. 108, 1067–1071.

Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A., Lamb, C.J., 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase in transgenic tobacco. Biotechniques 12, 807–812.

ARTÍCULO 6

Molecular characterization of a class I β-1,3-glucanase from *Vitis vinifera* cv. Cardinal.

Irene Romero, Carlos Fernandez-Caballero, Goñi Oscar, M. Isabel Escribano, Carmen Merodio, M. Teresa Sanchez-Ballesta

Plant Science **(aceptado)**

RESUMEN

Hemos analizado cómo el almacenamiento a baja temperatura (0ºC) y el tratamiento de 3 días con altos niveles de CO_2 (20% CO_2 +20% O_2) afectan la expresión génica de β-1,3-glucanasa de clase I (*Vcgns1*) y la actividad glucanasa en la uva de mesa roja (*Vitis vinifera* L. cv. Cardinal). Los resultados indican que el almacenamiento a 0ºC durante 3 días aumentó los niveles de mRNA de *Vcgns1* y la actividad β-1,3-glucanasa en la piel de uvas no tratadas. Sin embargo, la acumulación de los transcritos y el nivel de la actividad glucanasa fueron menores en la piel de uvas tratadas con CO_2 durante 3 días, así como cuando la fruta tratada fue transferida a aire. Utilizando la expresión heteróloga del cDNA de *Vcgns1* en *Escherichia coli*, vimos que codificaba un proteína con actividad glucanasa con un pH óptimo de 6 y temperatura óptima de 45 ºC, así como una alta estabilidad a 0ºC. Además, la proteína purificada VcGNS1 exhibió actividad crioprotectora *in vitro* para la enzima lábil a congelación L-lactato deshidrogenasa (LDH). Por el contrario, cuando fue medida la actividad de histéresis térmica (THA) de la proteína recombinante VcGNS1, usando calorimetría diferencial de barrido (DSC), los resultados indicaron que no presentó actividad anticongelante. La alta estabilidad de la proteína recombinante a 0ºC, y su actividad crioprotectora mostrada en este trabajo, sugiere que VcGNS1 podría participar en la respuesta de la uva de mesa para combatir las condiciones de bajas temperaturas.

MOLECULAR CHARACTERIZATION OF A CLASS I BETA-1,3-GLUCANASE FROM *Vitis vinifera* cv. Cardinal

Irene Romero[1], Carlos Fernandez-Caballero[1], Oscar Goñi, Mª Isabel Escribano, Carmen Merodio, Mª Teresa Sanchez-Ballesta*

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frio, IF-CSIC, Ciudad Universitaria, E-28040 Madrid, Spain.

*Author to whom correspondence should be addressed

[1]These authors contributed equally to this work.

Abstract

We have analyzed how low temperature storage (0 ºC) and a 3-day treatment of high CO_2 levels (20% CO_2 plus 20% O_2) affect class I β-1,3-glucanase (*Vcgns1*) gene expression and glucanase activity in the red table grape (*Vitis vinifera* L. cv. Cardinal). The results indicate that storage at 0 ºC for 3 days increased *Vcgns1* mRNA levels and β-1,3-glucanase activity in the skin of non-treated grapes. However, the accumulation of the transcripts, and the level of glucanase activity, were lower in the skin of grapes after 3 days of CO_2 treatment, as well as when treated fruit were transferred to air. By using heterologous expression of the *Vcgns1* cDNA in *Escherichia coli*, we showed that it encoded a protein with glucanase activity with an optimum pH and temperature of 6 and 45 ºC, respectively, and a high stability at 0 ºC. Furthermore, the purified VcGNS1 exhibited *in vitro* cryoprotective activity for the freeze labile L-lactate dehydrogenase (LDH) enzyme. In contrast, when the thermal hysteresis activity (THA) of the recombinant VcGNS1 was measured, using differential scanning calorimetry (DSC), the results indicated that it did not show antifreeze activity. The high stability of the recombinant protein at 0 ºC, and its cryoprotective activity shown in this work suggest that VcGNS1 may participate in the response of table grape to combat low temperature conditions.

Keywords: Table grapes; Carbon dioxide; Beta-1,3-glucanase; Gene Expression; Cryoprotective; Antifreeze

1. Introduction

Low temperature storage has always been used as the main method to extend the postharvest life of fruit and vegetables. Table grapes are not susceptible to injury at low (not freezing) temperatures, but their storage life is limited due to their high sensitivity to fungal attack. A number of studies have shown that exposure of plants to a moderate temperature stress not only induces resistance to severe stress of this kind, but can also improve tolerance to other stresses [1,2]. In previous studies we have observed that high CO_2 levels applied at low temperature during 3 days reduced total decay in table grapes, thereby maintaining fruit quality [3,4]. The responses of fruit to high CO_2 levels vary considerably among cultivars, and also depend on other environmental conditions of storage. Non-treated grapes responded to temperature shifts in the first stage of storage at 0 ºC, activating defense responses related to the phenylpropanoid pathway, whereas the application of high CO_2 levels reduced these responses [5].

In recent years, different studies have identified genes and metabolic pathways involved in the perception and signal transduction of plant responses to extreme temperatures (reviewed by [6]). Certain conserved mechanisms may still operate between chilling-sensitive and chilling-tolerant plants, and even between freezing and chilling tolerance [7]; but it is important to note that most of the studies were conducted in *Arabidopsis thaliana* during cold-acclimation to freezing, and little is known about the molecular basis of cold responses in agronomical important plants. Several studies have led to the identification of cold-responsive genes, many of which encode apoplastic antifreeze proteins (AFPs), late embryogenesis abundant proteins or pathogenesis related proteins (PRs), such as β-1,3-glucanases and chitinases [8].

β-1,3-glucanases (EC 3.2.1.39) hydrolyze the β-1,3-linked glucans found in the cell walls of higher plants, as well as in many fungi [9,10]. Glucanase gene expression may not only be modulated in response to many physiological processes in healthy plants [11,12,13], but also in response to biotic and abiotic stresses [12]. In winter rye, Hon et al. [14] observed that a cold-induced PR protein showed β-1,3-glucanase and cryoprotective activity. Likewise, a class I β-1,3-glucanase purified from tobacco protected thylakoids *in vitro*, isolated from spinach, against freeze-thaw damage [15].

Although isolation of β-1,3-glucanase genes have been reported in grapes [3,16,17], only limited information is presently available on the regulation of gene expression and the function of these proteins. The participation of β-1,3-glucanase in the defense of grapevine leaves against *Botrytis cinerea* has been shown [18,19]. However, in red table grapes we have observed that the efficacy of high CO_2 pretreatment in reducing total fungal decay is not mediated by the induction of class I β-1,3-glucanase and chitinase genes [3], indicating that factors other than fungal infection are involved in the induction of these genes in table grapes.

The aim of the present work was to analyze gene expression of *Vcgns1*, a class I β-1,3-glucanase, in red table grapes as a marker for changes in response to low temperature, and also to assess how high CO_2 levels (20%) modulated its transcript accumulation at 0 ^{o}C. As a part of our study to investigate the possible physiological role of class I β-1,3-glucanase in table grapes as a cryoprotectant, and/or an antifreeze, we also report on recombinant expression of *Vcgns1*.

2. Material and Methods

2.1. Plant Material

Table grapes (*Vitis vinifera* L. cv. 'Cardinal') were harvested at random in Camas (Sevilla, Spain) in July. After harvesting, the field-packaged bunches were transported to the laboratory where fruit were immediately forced-air precooled for 14 hours at –1 ºC. After cooling, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ±0.5 ºC and 95% relative humidity in two sealed neoprene containers of 1 m^3 capacity. One lot was stored under a normal atmosphere for 6 days (non-treated fruit), and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. The grapes were then transferred to air under the same conditions as non-treated fruit until the end of the storage period.

Ten clusters were sampled periodically, and berries from five clusters (approx. 300 g each cluster) were peeled, and the skin and pulp were frozen in liquid nitrogen, ground to a fine powder, and stored at –80 ºC until analysis.

2.2. RNA gel blot hybridization

Total RNA was extracted from the skin of grapes according to the method of Salzman et al. [20]. Samples of denatured total RNA (10 μg) from the skins were fractionated and blotted, as described in Romero et al. [3]. Equal loading was confirmed by membrane staining with methylene blue. The *Vcgns1* DNA probe was a random primer labeled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 ºC in 7% sodium dodecyl sulfate, 0.33 M phosphate buffer, pH 7.2, and 1 mM EDTA, then washed twice in

2 x SSC, 0.1% SDS at room temperature and twice in 0.1 x SSC, 0.1% SDS at 65 ºC, and exposed to Kodak X-Omat SX film at –80 ºC.

2.3. Production of the recombinant VcGNS1 protein in Escherichia coli

The cDNA fragment of VcGNS1 (GenBank accession number DQ267748) without the N-signal peptide and C-terminal extension was amplified using the forward primer (5'-ATT ATC CTC GAG GTG GGT GTA TGC TAT GGA ATG-3') and the reverse primer (5'-GGC GAA TTC AAA GTT GAT AGA GTA CTT CGG -3') containing *Xho*I and *Eco*RI restriction sites, respectively. The PCR conditions were 30 cycles of 40 s at 95 ºC, 40 s at 49 ºC and 40 s at 72 ºC. The amplified DNA fragments were digested with *XhoI* and *EcoRI* and ligated into the multicloning site of the pTrcHisA plasmid (Invitrogen, Carlstad, USA) previously digested with the same enzymes. The vector pTrcHisA-VcGNS1 was transformed into cells of *E. coli* strain TOP10, which were grown at 37 ºC in LB-medium with 50 µg mL-1 ampicillin and 0.8 mM glucose until the optical density at 600 nm reached 0.6, and then induced with 1 mM isopropyl-ß-D-thiogalactoside (IPTG). After 4 h of induction at 37 ºC the cells were harvested by centrifugation at 1760*g* for 20 min at 4 ºC and then frozen. Cells were re-suspended in lysis buffer (50 mM sodium phosphate monobasic, 500 mM sodium chloride, 10 mM imidazole, pH 8.0) and DNase1 RNase-Free (Roche); and then disrupted with 1 g of glass beads (150-252 µm, Sigma) in a FastPrep machine (FP120, Bio101, Savant). The cell extract was centrifuged at 1580*g* for 10 min at 4 ºC. The soluble recombinant His-tag VcGNS1 protein was incubated with Ni-NTA agarose resin (QIAexpress, Qiagen, Germany) for 1 h at 4 ºC with shaking, before the extract-resin mixture was loaded into a column. Contaminating proteins were eluted by washing the column with 8 mL of buffer (50 mM sodium phosphate monobasic, 500 mM sodium

chloride, 40 mM imidazole, pH 8.0). Bound VcGNS1 was eluted from the Ni-NTA column with elution buffer (5 x 1 mL 50 mM sodium phosphate monobasic, 500 mM sodium chloride, 300 mM imidazole, pH 8.0). The purified fusion protein was concentrated with buffer exchange into 50 mM Tris-HCl, pH 7.4 for enzymatic assays, into 20 mM sodium acetate buffer pH 5.0 for *in vitro* antifungal assays or doubled distilled water for *in vitro* cryoprotective and antifreeze assays by ultrafiltration on ultrafree biomax-5K (Millipore), and then stored at -20 ºC. Protein concentration was determined according to the Bradford method [21] using a protein-dye reagent (Bio-Rad) and bovine serum albumin as a standard.

2.4. Gel electrophoresis and protein blot analyses

Protein analysis was performed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [22], using Mini-Protean II Cell (Bio-Rad) equipment. Protein samples were prepared by boiling for 10 min in 2X SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, and 0.05 % bromophenol blue). For reducing conditions, samples were mixed with an equal volume of loading buffer supplemented previously with 10% β-mercaptoethanol. Protein bands were visualized by staining with Coomasie brilliant blue R-250. The molecular mass of the separated polypeptides was estimated by comparison with the mobility of pre-stained standard low molecular mass range proteins (Bio-Rad).

After standard PAGE was performed proteins were electro-transferred to PVDF membranes (Amersham) with a Mini Trans-Blot Cell (Bio-Rad). The Western blot was probed with a 1:5000 dilution of polyclonal anti-PR2 sera from tobacco, kindly provided by

Dr Fritig (Strasbourg, France), which were detected with a 1:5000 dilution of rabbit antiserum against IgG horseradish peroxidase conjugate (Amersham). The immuno-complexes were visualized using the enhanced chemiluminescence (ECL®) detection system (Amersham).

2.5. Protein extracts preparation

Ground frozen berry skin tissue (3 g FW) was homogenized in 10 mL of 100 mM sodium acetate buffer, pH 5.0, and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 35000*g* for 30 min. After removal of precipitated material the supernatant was adjusted to 95% saturation with ammonium sulphate, and centrifuged at 20000*g* for 20 min. The precipitate was re-suspended in 100 mM sodium acetate buffer, pH 5.0. All steps were carried out at 4 ºC.

2.6. β-1,3-glucanase activity assay

2.6.1. Protein extracts

β-1,3-glucanase activity was quantified measuring the reducing sugar released from the substrate *Laminaria digitata* laminarin (Sigma). 50 µL of extract was added to 300 µL of substrate (0.5 mg) in 100 mM sodium acetate buffer, pH 5.0. The reaction was stopped with 35 µL of NaOH after 8 h of incubation at 37 ºC and cooling on ice for 10 min. The reducing sugar was determined according to Dygert et al. [23]. D-glucose at concentrations from 2.5 to 120 µg mL^{-1} in buffer was used as a standard. Enzyme activity was defined as nmol glucose equivalents h^{-1} g FW^{-1}. Assays were determined in three replicated samples from three independent extractions.

2.6.2. Recombinant VcGNS1 protein

Optimum temperature value was determined by measuring the specific activity of 1 μg of purified VcGNS1 with 2 mg of *L. digitata* laminarin (Sigma) in 100 mM sodium acetate buffer, pH 6.0, at temperatures ranging between 0 ºC and 75 ºC. Optimum pH value was determined at 45 ºC by running the enzymatic assay with 1 μg of purified VcGNS1 in 2 mg of *L. digitata* laminarin (Sigma) in 100 mM phosphoric acid (pH 2.0), sodium acetate (pH 4.0-6.0), sodium phosphate dibasic (pH 7.0), Tris-HCl (pH 9.0) and sodium phosphate monobasic (pH 12) buffers. The mixtures were incubated with shaking for 60 min and the reducing sugars were measured as described above. The activity of VcGNS1 was recorded as the release of μmoles glucose equivalents min^{-1} mg $protein^{-1}$. Assays were determined in three replicated samples from three independent purifications of VcGNS1.

To determine the thermal and pH stability of the recombinant VcGNS1 the purified protein (1 μg) was incubated in 100 mM sodium acetate buffer, pH 6.0, without laminarin**,** for 1 h at temperatures between 0 ºC and 75 ºC and in the buffer at different pHs at 45 ºC, respectively. Subsequently, the residual activity was measured with 2 mg of laminarin as described above. Assays were determined in three replicated samples from three independent purifications of VcGNS1.

2.7. Kinetic parameters

The Michaelis-Menten constants K_m and k_{cat} were determined from the Hanes-Wolf representation of data obtained by measuring the initial rate of substrate hydrolysis. 1 μg of purified VcGNS1 was incubated at 45 ºC with increasing concentrations of laminarin (0-5 mg mL^{-1}) in 100 mM sodium acetate buffer, pH 6.0. After the reaction was stopped the

liberated glucose was measured as described above. The activation energy (E_a) was calculated by the method of Segel et al. [24], using the linear form of Arrhenius equation:

$$\log k = \frac{-E_a}{2.3R}\frac{1}{T} + \log A$$

in which R is the molar gas constant, T is the absolute temperature and k the specific reaction rate constant. For enzyme-catalyzed reactions log V_{max} can be plotted instead of log$k$.

The effect of δ-gluconolactone (Sigma) on the activity of the purified enzyme was measured as described previously Notario et al. [25].

2.8. Assay for cryoprotective activity

The *in vitro* cryoprotective activity was determined following the method described by Lin and Thomashow [26], with slight modifications. Lactate dehydrogenase (LDH, EC 1.1.1.23, Type V-S from rabbit muscle, Sigma, St. Louis, MO, USA) was diluted to 87.6 μg mL^{-1} in 20 mM potassium phosphate buffer, pH 7.5, and used as a freeze-labile stock enzyme solution. Samples, either VcGNS1 protein fractions or bovine serum albumin (BSA, Sigma, St. Louis, MO, USA), were diluted to varying concentrations and mixed with 2.364 μg of LDH from the stock solution. The resulting solution (300 μL) was frozen in liquid nitrogen for 30 s and then thawed at room temperature for 5 min. The freeze-thaw process was carried out three times and the residual LDH activity was then measured. LDH enzymatic activity was determined in aliquots of 30 μL of the mixtures in a final volume of 1.5 mL of the reaction assay buffer (80 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM pyruvate and 30 mM NADH), at room temperature. NAD^+ production was monitored as the

decrease of absorbance at 340 nm for 4 min. The data are showed as the percentage of the activity present in unfrozen controls. Assays were determined in three replicated samples.

2.9. Antifreeze activity measurement

The differential scanning calorimetry (DSC) was used to determine the antifreeze activity by measuring the thermal hysteresis activity (THA). DSC analysis was conducted using a differential scanning calorimeter (DSC822e, Mettler-Toledo Inc., Columbus, OH, USA) equipped with a liquid nitrogen cooling accessory. The instrument was calibrated using Indium and n-octane, and an empty aluminum pan was used as reference. An antifreeze protein (type III) from antarctic fish, kindly provided by Dr. De Vries (Illinois, USA), and BSA (AFP-free solution) were used as standards in order to make a comparison between solutions with and without antifreeze activity. Recombinant VcGNS1 protein (5 $mg \cdot mL^{-1}$) dissolved in double distilled water and 3-4 µl sample was weighed into 40 µL Mettler-Toledo coated aluminum pans. Pans with samples were immediately hermetically sealed and cooled from 25 ºC to -40 ºC at 10 ºC min^{-1}, frozen at this temperature for 5 min, and heated to melt (10 ºC min^{-1}). The samples were cooled at -30°C (10 ºC min^{-1}) for 5 min, and warmed up slowly to various partial-melt temperatures (0.5 ºC min^{-1}). To allow ice-protein interaction, samples were held at this temperature (T_h, hold temperature) for 10 min before being recooled at the same rate of 0.5 ºC min^{-1} to -25 ºC, during which the onset temperature of crystallization (T_c) was recorded using STARe software (Mettler-Toledo). THA was defined as the difference between T_h and T_c, $THA = T_h - T_c$.

2.10. Statistical analysis

The data from at least three replicates per sample were subjected to analysis of variance (One-way ANOVA) using the LHD test to determine the level of significance at $P \leq 0.05$ (Statgraphics Plus version 5.1, STSC, Rockville, Md.).

3. Results

3.1. Effect of low temperature and high CO_2 on Vcgns1 mRNA levels and β-1,3-glucanase activity

In the skin of non-treated grapes low temperature sharply increased the levels of the transcript after 3 and 6 days of storage (Fig. 1A). In contrast, high CO_2 levels applied during 3 days at 0 ºC decreased the accumulation of the *Vcgns1* mRNA, but levels increased slightly when treated grapes were transferred to air for 3 days, although the levels were always lower than in the non-treated. Glucanase activity increased significantly after 3 days at 0 ºC in non-treated grapes, and then decreased after 6 days (Fig. 1B). The increase of glucanase activity after 3 days of CO_2 at 0 ºC treatment was lower than in non-treated grapes, increasing slightly thereafter.

3.2. Expression and purification of VcGNS1

The full-length of the VcGNS1 was previously isolated from the skin of table grape using RT-PCR and 5'-3' RACE strategies [3]. The predicted amino acids of VcGNS1 cDNA shared homology with basic class I β-1,3-glucanases containing a N-terminal signal peptide cleavage site located between residues 22 (Lys) and 23 (Ser), and a C-terminal

extension of 23 amino acids with a *N*-glycosylation site, and required for targeting the protein to the vacuole [27,28].

To determine whether the VcGNS1 cDNA encoded a protein with glucanase activity, and to perform *in vitro* cryoprotective and antifreeze assays, the cDNA fragment corresponding to the mature protein (without the putative N-terminal transit peptide and the C-terminal extension, amino acid residues 23 to 338) was expressed in TOP10 *E. coli* cells as a pTrcHisA-VcGNS1 fusion protein. The VcGNS1 protein was induced in the presence of 1 mM IPTG at 37 ºC for 4 h and purified with a Ni-NTA affinity column. After purification, gel electrophoresis of both reduced and unreduced VcGNS1 yielded a single band of 43 and 40 kDa respectively (Fig. 2A), suggesting that the native protein occurs as a monomer. Western blot analysis with an antiserum against tobacco PR2 showed a positive band with the same molecular mass (Fig. 2B).

3.3. Effects of temperature and pH on enzyme activity and stability

The enzymatic activity of the recombinant β-1,3-glucanase was measured over a pH (2-12) and temperature (0-70 ºC) range by assaying the production of reduced sugars from laminarin (Fig. 3). The pH optimum for recombinant VcGNS1 was 6, decreasing sharply at pH values above 6.5 or below 5.5. The temperature optimum was 45 ºC, but the recombinant VcGNS1 retained some hydrolytic activity at different temperatures assayed. In terms of stability, the recombinant protein was able to catalyze the hydrolysis of laminarin over a wide range of pH, 2 to 9, and temperature, 0 ºC to 45 ºC, being inactive at pH 12 and at temperatures higher than 60 ºC. Interestingly, the recombinant protein showed

the optimum stability at 0 ºC, reaching values significantly higher to those achieved when the activity was measured at 45 ºC.

3.4. Kinetic properties

The recombinant β-1,3-glucanase obeys Michaelis-Menten kinetic. A linear curve was obtained from the Hanes-Wolf plot for the hydrolysis of laminarin at concentrations of 0-5 mg/mL, yielding an apparent K_m value of 0.78 mg/mL which, corresponds to 0.195 mM. The catalytic rate constant, k_{cat}, is 7.96 $s^{-1}$and the specificity or catalytic efficiency factor, k_{cat}/K_m, 40.82 s^{-1} mM^{-1}. The calculated E_a was 19.95 kJ/mol for laminarin hydrolysis (Table 1). Moreover, the VcGNS1 did not show inhibition with δ-gluconolactone (1 to 20 mM), a potent inhibitor of exo-β-glucanases at low concentrations (Notario et al, 1976), indicating an endotype mode of action for the recombinant enzyme (data not shown).

3.5. Cryoprotective activity of recombinant VcGNS1

The ability of recombinant β-1,3-glucanase to preserve LDH activity following freeze-thaw cycles was compared with the level of cryoprotection provided by BSA. The cryoprotective activity of recombinant VcGNS1 and BSA was then assessed by determining whether they could protect LDH against freeze inactivation. Without the addition of a cryoprotectant three freeze-thaw cycles resulted in a reduction of more that 80% in LDH activity (Fig. 4). The addition of VcGNS1 or BSA in protein:enzyme molar ratios from 1 to 4 significantly protected LDH activity. Recombinant VcGNS1 was shown to be less efficient than BSA in protecting LDH at molar ratios from 1 to 3. At a molar ratio of 4, VcGNS1 was as efficient as BSA in protecting LDH activity against freeze

inactivation, maintaining around 70% of initial LDH activity. On the contrary, the addition of sucrose at similar concentrations failed to protect LDH under identical freeze-thawing conditions.

3.6. Thermal hysteresis activity of recombinant VcGNS1

DSC curves of the recrystallization of different partially melted protein-water systems are presented in Fig. 5. A delay of 0.4 ºC in the onset temperature of refreezing was observed in a partially melted AFP system. In recombinant VcGNS1 solution, recrystallization of the melted part started immediately after the temperature dropped, and the exothermic peak appeared without delay. This indicated that the VcGNS1, like AFP-free BSA solution, had no thermal hysteresis effect.

4. Discussion

Grapes are classified as a chilling tolerant fruit, and are normally injured at temperatures below -20 ºC. Previous results indicated that table grapes cv. Cardinal could be sensitive to temperature shifts at 0 ºC, and that high CO_2 levels applied as a pretreatment could regulate these responses [4]. To further identify the mechanisms associated with the response of table grapes at 0 ºC, and to determine whether high CO_2 levels could modulate them, we have analyzed changes in β-1,3-glucanase gene expression in the first stage of storage. There have been several previous reports of PR proteins induced by low temperature. In winter rye, one of the PR proteins induced in response to low temperature showed β-1,3-glucanase and cryoprotective activity [14]. In citrus, Sanchez-Ballesta et al. [29] observed that changes in gene expression of a class III β-1,3-glucanase were linked to postharvest chilling-induced cell damage. In tomato exposed at chilling temperature a class

II β-1,3-glucanase mRNA was induced [30]. In previous work, we have isolated a class I β-1,3-glucanase (*Vcgns1*) cDNA from the skin of table grape [3]. The levels of the *Vcgns1* transcript increased in non-treated grapes after 12 days at 0 ºC, but we did not find any relation between *Vcgns1* gene expression and fungal attack, indicating that factors other than fungal infection are involved in *Vcgns1* gene expression in table grapes. In this work we observed that low temperature by itself has a clear effect on *Vcgns1* gene expression and glucanase activity. Thus, after 3 days at 0 ºC a sharp increase in the level of *Vcgns1* mRNA was observed in the skin of non-treated grapes, maintaining the level after 6 days. In contrast, 3 days CO_2-treatment reduced the accumulation of the transcript observed at 0 ºC, with a slight increase in the levels when treated grapes were transferred to air (Fig. 1A). Likewise, although glucanase activity increased in response to low temperature, the increase was higher after 3 days at 0 ºC in the non-treated grapes. Previously, similar results were observed in non-treated grapes, where phenylpropanoid gene expression, total anthocyanin accumulation and antioxidant activity were activated during the first stage of storage at 0 ºC, whereas the application of high CO_2 treatment reduced these responses [4]. These results reinforce the idea that high CO_2 levels applied at 0 ºC as a pretreatment of 3 days can modulate the responses induced in non-treated grapes in the first stage of storage at 0 ºC.

To determine whether the *Vcgns1* cDNA isolated from table grape encoded a protein with glucanase activity, and to analyze its functionality, the protein was produced by the pTrcHis expression system in *E. coli.* The purified recombinant protein showed a single protein band in SDS-PAGE. The molecular mass of the recombinant VcGNS1 was 40 kDa, and this value agreed with the molecular mass calculated from the deduced polypeptide. The purified VcGNS1 showed β-1,3-glucanase activity for laminarin

hydrolysis with a pH optimum higher than other plant β-1,3-glucanase enzymes [31,32], but with similar optimum temperature. The VcGNS1 protein was relatively more thermostable than the β-1,3-glucanases from *Boscia senegalensis* [32] and barley [33], being active over a wide range of temperatures. The recombinant glucanase displayed 27% of relative activity at 0 ºC and, interestedly at this temperature, VcGNS1 showed a higher thermostability after 1 h without substrate. The Ea for VcGNS1 is lower than values obtained for other carbohydrate hydrolases [32] and constant over the studied temperature range, indicating that the enzyme does not undergo major structural changes within this range. In winter rye glucanases presented the maximal activity at temperatures around 30 ºC, and there was clear evidence of activity at zero and subzero temperatures [34]. However, to our knowledge, this is the first report where a β-1,3-glucanase from fruit showed low values of Ea and hydrolytic activity at cold temperatures. Kinetics analyses of the recombinant VcGNS1 during hydrolysis of laminarin allowed the calculation of a K_m value (0.195 μM) which is substantially higher than that reported for β-1,3-glucanases from other plants [32,35]. However, the turnover number (k_{cat} 7.96 s^{-1}) was significantly lower than values reported for plant glucanases [33,35]. Furthermore, VcGNS1 could behave as an endo-splitting hydrolase because of the non inhibitory effect of δ-gluconolactone on the enzymatic activity observed in this work (data not shown). In this respect, this enzyme is comparable to other plant β-1,3-glucanases isolated [32,33,36,37].

It has been reported that proteins identified as PRs that accumulate in winter cereals during cold acclimation function as antifreeze proteins, by inhibiting extracellular ice growth under freezing temperatures [14]. Yaish et al. [34] support the hypothesis that recombinant winter rye glucanases have evolved to inhibit the formation of large ice

crystals, in addition to the capacity to degrade glucans at low temperature. In contrast, the recombinant VcGNS1 did not show antifreeze activity when the THA was determined by DSC. In spinach and cabbage leaves β-1,3-glucanase proteins were accumulated during cold acclimation, and glucanase of class I had potent cryoprotective activity [15]. Likewise, a PR-5 protein from groundnut showed *in vitro* cryoprotective activity against LDH [38]. However, this appears not to be a general effect of stress-induced PR proteins. A tobacco class I chitinase did not demonstrate any cryoprotective effects [15]. In chilling-sensitive mandarins the recombinant class III β-1,3-glucanase protein, likewise, showed glucanase but not cryoprotective activity [29]. In this study we demonstrate that VcGNS1 had cryoprotection activity. Assay for freeze-thawing enzyme inactivation was done with LDH, because of its sensitivity to different stresses. A comparison of the cryoprotection conferred by VcGNS1 revealed that it was similar to that of BSA, which is a known cryoprotectant [39]. Similar results have been observed with WAP18, a PR10 from mulberry [40]; COR15am, a LEA-like protein from *Arabidopsis* [41]; and winter rye apoplastic proteins [42], which in all situations exhibited a similar value to BSA. In contrast the cryoprotective activities of COR85 and PCA60, cold-induced dehydrins, were found to be around four or two-fold higher than that for BSA [43,44]; but CrCOR15, a dehydrin from citrus fruit revealed a cryoprotective activity similar to BSA [45]. The percentage of polar residues in VcGNS1 is around 40% as occur in a PR-10 protein such as WAP18 [40]. These authors indicated that the lower-cryoprotective activity of WAP18, in comparison with other cryoprotectants, may be partially related to the lower-ratio of polar residues in the surface of the protein.

In conclusion, we have identified a class I β-1,3-glucanase from table grapes that displays glucanase activity as well as *in vitro* cryoprotective but not antifreeze activity.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Education and Science (AGL2005-04502/ALI) and Comunidad Autonoma de Madrid (200670M050). I.R. and MT.S.-B. were supported by a postgraduate and postdoctoral I3P contract from the CSIC, respectively. C. F-C. and O. G. were supported by a predoctoral fellowship from MEC, Spain. The authors are grateful to B. Fritig (Strasbourg, France) for his generosity in providing PR-2 antisera, to Frutaria SAT for providing tables grapes and to Agrotecnica for industrial assistant.

References

[1] C. Bowler, R. Fluhr, The role of calcium and activated oxygens as signals for controlling cross-tolerance, Trends Plant Sci. 5 (2000) 241-246.

[2] W.X. Wang, B. Vinocur, A. Altaman, Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance, Planta 218 (2003) 1-14.

[3] I. Romero, M.T. Sanchez-Ballesta, R. Maldonado, M.I. Escribano, C. Merodio, Expression of a class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO_2 pretreatment, Postharvest Biol. Technol. 41 (2006) 9-15.

[4] M.T. Sanchez-Ballesta, J.B. Jiménez, I. Romero, J.M. Orea, R. Maldonado, A. González-Ureña A, M.I. Escribano, C. Merodio, Effect of high CO_2 pretreatment on quality, fungal decay and molecular regulation of stilbene phytoalexin biosynthesis in stored table, Postharvest Biol. Technol. 42 (2006) 209–16.

[5] M.T. Sanchez-Ballesta, I. Romero, J.B. Jiménez, J.M. Orea, A. González-Ureña A, M.I. Escribano, C. Merodio, Involvement of phenylpropanoid pathway in the response of table grapes to low temperature and high CO2 levels. Postharvest Biol. Technol. 46 (2007) 29-35.

[6] D.Y. Sung, F. Kaplan,K.J. Lee, C.L. Guy, Acquired tolerance to temperature extremes, Trends Plant Sci. 8 (2003) 179-187.

[7] T.H. Hsieh, J.T. Lee, P.T. Yang, L.H. Chiu, Y.Y. Charng, Y.C. Wang, M.T. Chan, Heterology expression of the Arabidopsis C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato, Plant Physiol. 129 (2002) 1086-1094.

[8] M.F. Thomashow, Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 571-599.

[9] J. Wessels, J. Sietsma, Fungal cell walls: a survey, in: W. Tanner, F. Loewus (Eds.), Encyclopedia of Plant Physiology, New Series, Vol. 13 B, Plant Carbohydrates II, Springer-Verlag, Berlin, 1981, pp. 352–394.

[10] Y. Takeuchi, M. Yoshikawa, G. Takeba, K. Tanaka, D. Shibata, O. Horino, Molecular cloning and ethylene induction of mRNA encoding a phytoalexin elicitor-releasing factor, β-1,3-glucanase, in soybean, Plant Physiol. 93 (1990) 673-682.

[11] H. Stieglitz, Role of beta-1,3-glucanase in postmeiotic microspore release, Dev Biol. 57 1977) 87-97.

[12] C.R. Simmons, Physiology and molecular-biology of plant 1,3-beta-d-glucanases and 1,3 1,4-beta-d-glucanases, Critic. Rev. Plant. Sci. 13 (1994) 325-387.

[13] R. Vöngeli-Lange, C. Fründt, C.M. Heart, F. Nagy, F.Jr. Meins, Developmental, hormonal, and pathogenesis-related regulation of the tobacco class I beta-1,3-glucanase B promoter, Plant Mol. Biol. 25 (1994) 299-311.

[14] W.C. Hon, M. Griffith, A. Mlynarz, Y.C. Kwok, D.S.C. Yang, Anitfreeze proteins in winter rye are similar to pathogenesis related proteins, Plant Physiol. 109 (1995) 878-889.

[15] D.K. Hincha, F.Jr. Meins, J.M. Schmitt, β-1,3-glucanase is cryoprotective in vitro and is accumulated in leaves during cold acclimation, Plant Physiol. 114 (1997) 1077-1083.

[16] E. Kraeva, C. Tesniere, N. Terrier, C. Romieu, F.X. Sauvage, J. Bierne, A. Deloire, Transcription of a beta-1,3-glucanase gene in grape berries in a late developmental period, or earlier after wounding treatments, Vitis 37 (1998) 107-111.

[17] A.K. Jacobs, I.B. Dry, S.P. Robinson, Induction of different pathogenesis-related cDNAs in grapevine infected with powdery mildew and treated with ethephon, Plant Pathol. 48 (1999) 325-336.

[18] J.P. Derckel, L. Legendre, J. Audran, B. Haye, B. Lambert, Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced in leaves by salicilic acid are constitutively expressed in other tissues, Plant Sci. 119 (1996) 31-37.

[19] A.S. Renault, A. Deloire, J. Bierne, Pathogenesis-related proteins in grapevines induced by salicylic acid and *Botrytis cinerea*, Vitis 35 (1996) 49-52.

[20] R.A. Salzman, T. Fujita, K. Zhu-Salzman, P.M. Hasegawa, R.A. Bressan, An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates, Plant Mol. Biol. Rep. 17 (1999) 11-17.

[21] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685.

[23] S. Dygert, L.H. Li, D. Florida, J.A. Thomas, Determination of reducing sugar with improved precision, Anal. Biochem. 13 (1965) 367-374.

[24] V. Segel, J. Pelleg, D. Fuks, S. Dorfman, A model describing the curved arrhenius plots in self-diffusion of metals, Phys. Stat. Sol. (a) 140 (1993) 363-368.

[25] V. Notario, T.G. Villa, J.R. Villanueva, Purification of an exo-beta-D-glucanase from cell-free extracts of *Candida utilis*, Biochem. J. 159 (1976) 555-562.

[26] C. Lin, M.F. Thomashow, A cold-regulated *Arabidopsis* gene encodes a polypeptide having potent cryoprotective activity. Biochem. Biophys. Res. Commun. 183 (1992) 1103-1108.

[27] H. Shinshi, Wenzler, H., J.M. Neuhaus, G. Felix, J. Hofsteenge, Jr.F. Meins, Evidence for N- and C-terminal processing of a plant defense-related enzyme: primary structure of tobacco prepo-β-1,3-glucanase, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 5541-5545.

[28] F. Meins, J.M. Neuhaus, C. Sperisen, J. Ryals, The primary structure of plant pathogenesis-related glucanohydrolases and their genes, in: T. Boller, F.Jr. Meins, (Eds.), Genes Involved in Plant Defense, Vienna, Springer-Verlag, New York, 1992, 245-282.

[29] M.T Sanchez-Ballesta, M.J. Gosalbes, M.J. Rodrigo, A. Granell, L. Zacarias, M.T. Lafuente, Characterization of a β-1,3-glucanase from citrus fruit as related to chilling-induced injury and ethylene production, Postharvest Biol. Technol. 40 (2006) 133-140.

[30] C.K. Ding, C.Y. Wang, K.C. Gross, D.L. Smith, Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit, Planta 214 (2002) 895-901.

[31] N. Churngchow, A. Suntaro, R. Witisuwannnakul, beta-1,3-glucanase isozymes from the latex of hevea-brasiliensis, Phytochem. 39 (1995) 505-509.

[32] M.H. Dicko, M.J. Searle-van Leeuwen, A.S. Traore, R. Hilhorst, G. Beldman, Polysaccharide hydrolases from leaves of *Boscia senegalensis*: properties of endo-(1→3)-beta-D-glucanase, Appl. Biochem. Biotechnol. 94 (2001) 225-241.

[33] M. Hrmova, G.B. Fincher, Purification and properties of three (1→3)-beta-D-glucanase isoenzymes from young leaves of barley (*Hordeum vulgare*), Biochem. J. 289 (1993) 453-461.

[34] M.W.F. Yaish, A.C. Doxey, B.J. McConkey, B.A. Mpffatt, M. Griffith, Cold-active winter rye glucanases with ice-bindung capacity. Plant Physiol. 141 (2006) 1149-1472.

[35] T. Akiyama, N. Shibuya, M. Hrmova, GB. Fincher, Purification and characterization of a (1→3)-beta-D-glucan endohydrolase from rice (*Oryza sativa*) bran, Carbohydr. Res. 297 (1997) 365-374.

[36] R. Vogelsang, W. Barz, Purification, characterization and differential hormonal regulation of a beta-1,3-glucanase and two chitinases from chickpea (*Cicer arietinum* L.), Planta 189 (1993) 60-69.

[37] Y. Morohashi, H. Matsushima, Development of beta-1,3-glucanase activity in germinated tomato seeds, J. Exp. Bot. 51 (2000) 1381-1387.

[38] R.S. Dave, R.K. Mitra, A low temperature induced apoplastic protein isolated from *Arachis hypogea*, Phytochem. 4 (1998) 2207-2213.

[39] T. Tamiya, N. Okahashi, R. Sakuma, T. Aoyama, T. Akahane, J.J. Matsumoto, Freeze denaturation of enzymes and its prevetion with additives, Cryobiol. 22 (1985) 446-456.

[40] N. Ukaji, C. Kuwabara, D. Takezawa, K. Arakawa, S. Fujicawa, Accumulation of pathogenesis related (PR) 10/Bet v 1 protein homologues in mulberry (*Morus bobycis* Koidz) tree during winter, Plant Cell Environ. 27 (2004) 1112-1121.

[41] N.N. Artus, M. Uemura, P.L. Steponkus, S.J. Gilmour, C. Lin, M.F. Tomashow, Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 13404-13409.

[42] M. Griffith, C. Lumb, S.B. Wiseman, M. Wisniewski, R.W. Johnson, A.G. Marangoni, Antifreeze proteins modify the freezing process in planta, Plant Physiol. 138 (2005) 330-340.

[43] T. Kazuoka, K. Oeda, Purification and characterization of cor85-oligomeric complex from cold-acclimated spinach, Plant Cell Physiol. 35 (1994) 601-611.

[44] M. Wisniewski, R. Webb, R. Balsamo, T.J. Close, X.M. Yu, M. Griffith, Purification, immunolocalization, cryoprotective, and antifreeze activity of PCA60: A dehydrin from peach (*Prunus persica*), Physiol. Plant. 105 (1999) 600-608.

[45] M.T. Sanchez-Ballesta, M.J. Rodrigo, M.T. Lafuente, A. Granell, L. Zacarias, Dehydrin from citrus, which confers in vitro dehydration and freezing protection activity, is constitutive and highly expressed in the flavedo of fruit but responsive to cold and water stress in leaves, J. Agric. Food Chem. 52 (2004) 1950-1957.

Figure Captions

Fig. 1. Effect of low temperature and high CO_2 pretreatment on *Vcgns1* mRNA accumulation (A) and β-1,3-glucanase activity (B) in the skin of 'Cardinal' table grapes (A) 10 μg of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *Vcgns1* probe. The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. (B) Time courses of β-1,3-glucanase activity in the skin of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 ºC. Error bars represent S.E (n=9). Values labeled with the same letter are not different at the 5% significance level.

Fig. 2. (A) Recombinant VcGNS1 protein was analysed by SDS-PAGE (12% polyacrylamide) and stained by Coomassie blue. Lanes 2 and 3 were loaded with 2 μg of reduced (with 10 % β-mercaptoethanol) and unreduced protein. Lane 1 was loaded with low molecular mass reference proteins and sizes are indicated on the left. (B) Immunoblot of a similar gel with unreduced VcGNS1. The blot was incubated with polyclonal anti-PR2 sera from tobacco.

Fig. 3. Effect of pH and temperature on the activity (–●–) and stability (–○–) of recombinant VcGNS1. Optimum temperature was determined by measuring the specific activity of 1 μg of purified VcGNS1 in a range of temperatures from 0 ºC to 80 ºC at pH 6.0. Optimum pH was determined by measuring the activity at 45 ºC in the range of pH 2 to 12. Stability was assessed by measuring the residual activity after 1 h of incubation at different pH and temperatures. For all experiments, laminarin degradation was measured

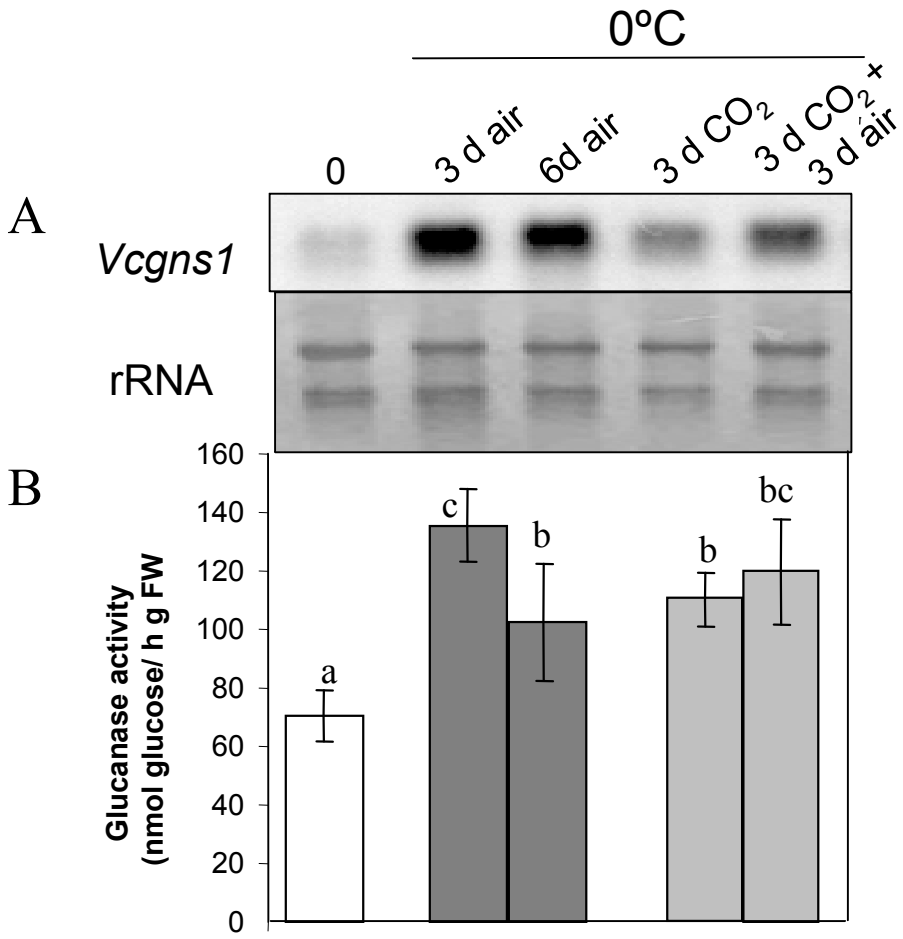
under standard assay conditions. Values presented are expressed in relative activity (percent of the greatest activity detected). Error bars represent S.E (n=9).

Fig. 4. Cryoprotection of LDH by recombinant VcGNS1. A LDH solution was frozen with different concentrations of VcGNS1, BSA or sucrose. The samples were thawed at room temperature and the LDH activity was measured. The relative activity represents the amount of LDH activity remaining after a freeze-thaw treatment as a percentage of the control enzyme activity. Error bars represent S.E (n=3).

Fig. 5. Refreezing DSC curves (0.5 ºC min^{-1}) of partially melted protein-water systems. A) AFP-III B) BSA C) recombinant VcGNS1.

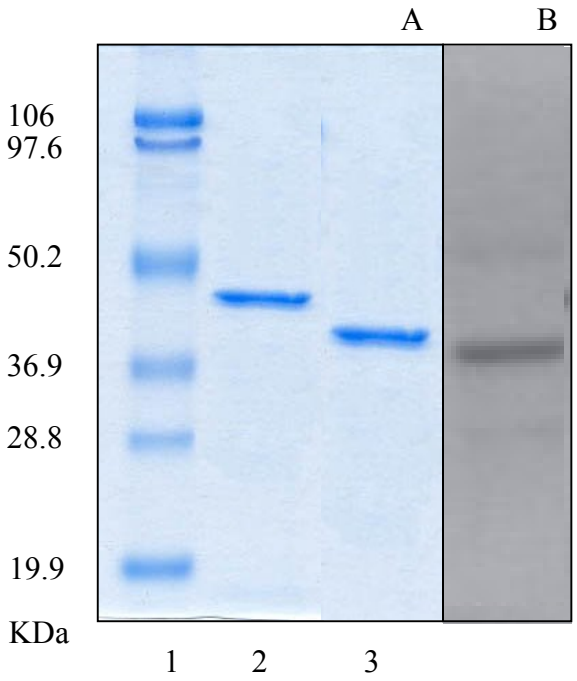
Figure(s)

Figure 1



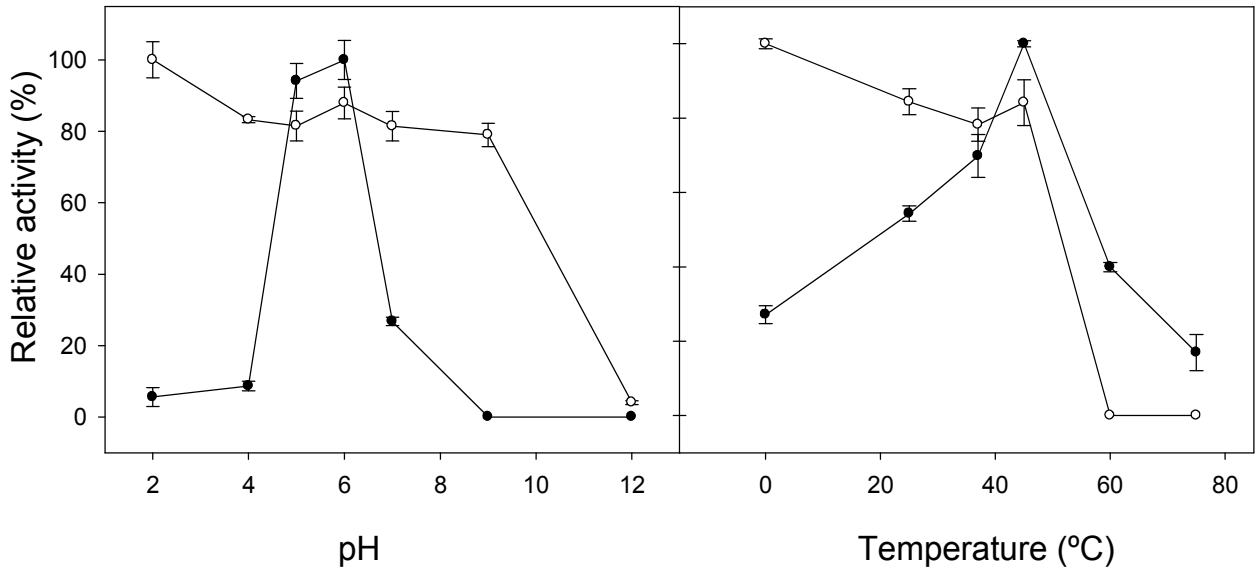
Figure(s)

Figure 2



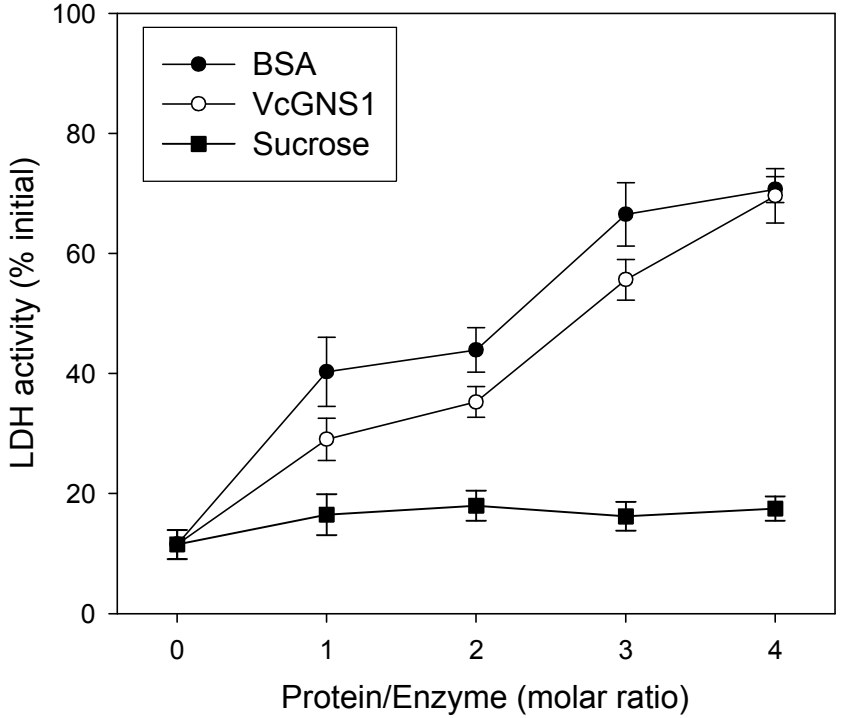
Figure(s)

Figure 3



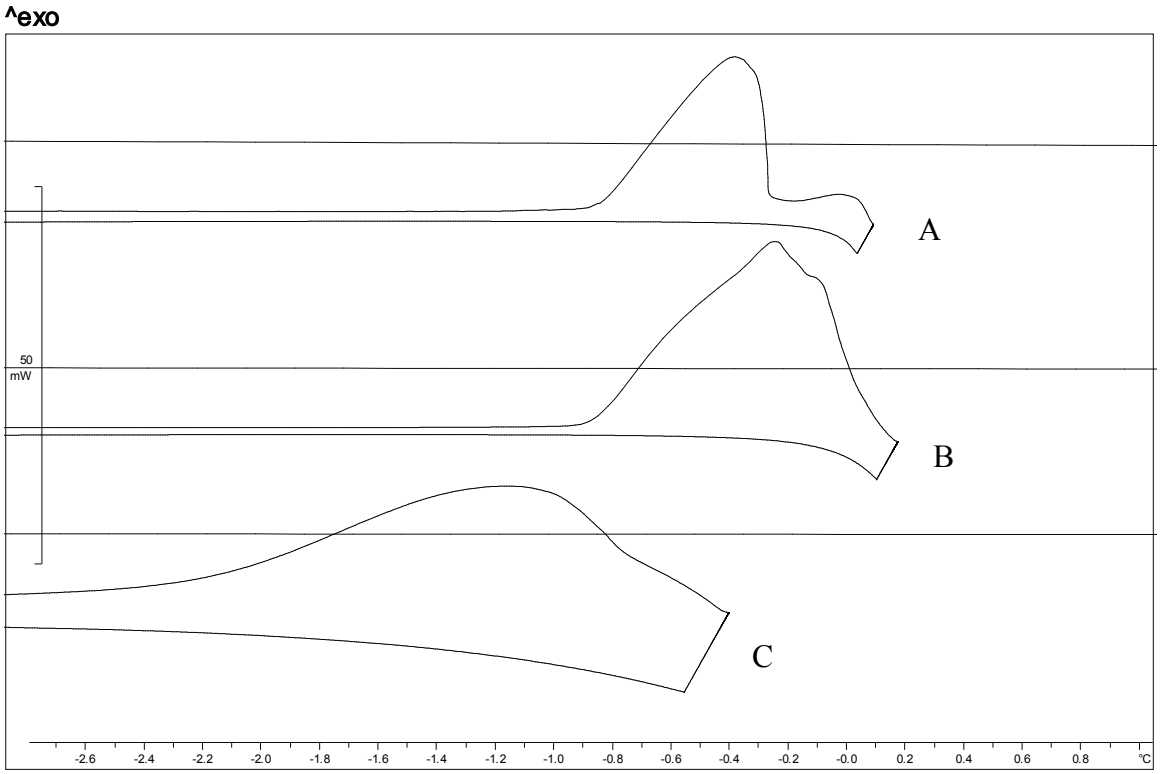
Figure(s)

Figure 4



Figure(s)

Figure 5



Table(s)

Table 1. Kinetic properties of recombinant VcGNS1 using *L. digitata* laminarin.

Spact (μmol min^{-1} mg^{-1})[a]	9.70
K_m	
(mg mL^{-1})	0.78
(mM)	0.195
k_{cat} (s^{-1})[b]	7.96
k_{cat}/K_m (s^{-1} mM^{-1})	40.82
E_a (kJ mol^{-1})[c]	19.95

[a] Specific activity.
[b] Catalytic constant rate (k_{cat}) = V_{max}/[E], where [E] = 0.025 nM.
[c] Activation energy determined between 0 and 45 ºC.

ARTÍCULO 7

Functionality of a class I chitinase from skin of grape berries.

Irene Romero, Carlos Fernandez-Caballero, M. Isabel Escribano, Carmen Merodio, M. Teresa Sanchez-Ballesta.

Postharvest Biology and Technology. **Short Communication (pendiente de enviar)**

RESUMEN

Hemos analizado cómo el almacenamiento a 0ºC y altos niveles de CO_2 (20% CO_2 + 20% O_2) afectan a la expresión génica de una quitinasa de clase I (*VcCHIT1b*) en la piel de uvas de mesa (*Vitis vinifera* L. cv. Cardinal). El almacenamiento a bajas temperaturas durante 3 días indujo la expresión génica de *Vcchit1b* en uvas no tratadas. Por el contrario, el tratamiento de 3 días de CO_2 redujo el incremento en la acumulación de transcritos. Nosotros hemos sobreexpresado el cDNA de quitinasa de clase I en *Escherichia coli.* Debido a que la proteína se produjo en cuerpos de inclusión, la proteína fue solubilizada y replegada. La proteína recombinante mostró inhibición antifúngica contra *Botrytis cinerea* en estudios realizados con placas de agar. Además la proteína purificada VcCHIT1b exhibió una gran actividad crioprotectora para la enzima lábil a la congelación L-lactato deshidrogenase (LDH). Estos resultados sugieren que VcCHIT1b podría participar en la respuesta de las uvas de mesa para combatir las condiciones de bajas temperaturas y el ataque fúngico.

FUNCTIONALITY OF A CLASS I CHITINASE FROM SKIN OF GRAPE BERRIES

Irene Romero, Carlos Fernandez-Caballero, Mª Isabel Escribano, Carmen Merodio, Mª Teresa Sanchez-Ballesta*

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frio, IF-CSIC, Ciudad Universitaria, E-28040 Madrid, Spain.

* Author to whom correspondence should be addressed. Telephone 34-91-5492300. Fax 34-91-5493627. E-mail: mballesta@if.csic.es

Abstract

We have analyzed how low temperature (0 ºC) storage and high CO_2 (20% CO_2 + 20% O_2) levels affect class I chitinase (*VcCHIT1b*) gene expression in the skin of table grapes (*Vitis vinifera* L. cv. Cardinal). Low temperature storage for 3 days induced *Vcchit1b* gene expression in non-treated grapes. By contrast, the 3-day treatment of high CO_2 levels reduced the increase in the transcript accumulation. We have overexpressed the chitinase class I cDNA in *Escherichia coli*. Since the protein was produced as insoluble inclusion bodies, the protein was solubilized and refolded. The recombinant protein showed antifungal inhibition against *Botrytis cinerea* in agar plates. Furthermore, the purified VcCHIT1b exhibited potent *in vitro* cryoprotective activity for the freeze labile L-lactate dehydrogenase (LDH) enzyme. These results suggest that VcCHIT1b may participate in the response of table grapes to combat low temperature conditions and fungal attack.

Keywords: Table grapes; Carbon dioxide; Chitinase; Gene Expression; Cryoprotective; Antifungal.

1. Introduction

Decay caused by *Botrytis cinerea* is responsible for most of the postharvest losses of table grapes during storage at low temperature (*Vitis vinifera* L.). This mold is controlled mainly by extensive use of fungicides. However, fungicides and chemical treatments may cause damage to grape berries if used excessively, and some consumers develop allergic reactions. Alternative gaseous methods such as controlled atmospheres and modified atmosphere packaging controlled table grapes postharvest decay (Crisosto et al., 2002; Artés-Hernández et al., 2004). In previous works, we showed the efficacy of a 3-day pretreatment with high CO_2 levels in maintaining the quality of the table grapes, and controlling the total decay (Romero et al., 2006). Likewise, high CO_2 treatment reduced the defence responses related to phenylpropanoid metabolism of table grapes in response to temperature shifts in the first stage of storage at 0ºC (Sanchez-Ballesta et al., 2007). However, our previous studies indicated that the efficacy of high CO_2 pretreatment reducing total decay is not mediated by the induction of pathogenesis-related proteins (PRs) genes, such as class I chitinase and b-1,3-glucanase (Romero et al., 2006), indicating that factors other than fungal infection are involved in the induction of these genes in table grapes.

Chitinases catalyze the hydrolysis of chitin, a linear polymer of β-1,4-linked N-acetylglucosamine residues, which is a major structural component of the cell walls of many fungi. In plants, chitinases were induced upon invasion by pathogens and by a variety of abiotic stress factors (Collinge, 1993). Transgenic plants overexpressing chitinase and β-1,3-glucanase genes show enhanced resistance to fungal infection (Grison et al., 1996). A class II chitinase gene had important roles in bermudagrass in response to low temperature (de los Reyes et al., 2001). Likewise, class I and class II chitinases from winter rye were induced by low temperature and showed antifreeze

activity (Yeh et al., 2000). Although it has been reported the isolation of class I chitinase genes in grapes (Busam et al., 1997; Robert et al., 2002); only limited information is presently available on the regulation of gene expression in response to fungal attack. The aim of the present work was to analyze expression of a class I chitinase gene in red table grapes exposed to low temperature and also assess how high CO_2 levels modulated its transcript accumulation. Likewise, we have investigated the possible physiological role of this protein in table grapes as antifungal and/or cryoprotectant, by the heterologous expression in *Escherichia coli*.

2. Material and Methods

Table grapes (*Vitis vinifera* L. cv. 'Cardinal') were harvested at random in Camas 5 (Sevilla, Spain) in July. After harvesting, the field-packaged bunches were transported to the laboratory where fruit were immediately forced-air precooled for 14 hours at –1 ºC. After cooling, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ±0.5 ºC and 95% relative humidity in two sealed neoprene containers of 1 m3 capacity. One lot was stored under a normal atmosphere for 6 days (non-treated fruit), and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. The grapes were then transferred to air under the same conditions as non-treated fruit until the end of the storage period. Berries from five clusters (approx. 300 g each cluster) were peeled, and the skin and pulp were frozen in liquid nitrogen, ground to a fine powder, and stored at –80 ºC until analysis.

Total RNA extraction and northern blots were performed as described by Romero et al. (2006). Samples of denatured total RNA (10 μg) from the skin of grapes

were fractionated and blotted. Equal loading was confirmed by membrane staining with methylene blue. The *Vcchit1b* DNA probe was random primer labeled with α^{32}P-dCTP

The cDNA fragment of *Vcchit1b* (GenBank accession number DQ267094) without the N-signal peptide was amplified using forward primer (5'- ATT ATC GGA TCC GAG CAA TGT GGA GGG CAA GCT-3') and reverse primer (5'-GGC GAA TTC TTA GAT GGT GTC CAG CAG GAG-3') containing *Bam*HI and *Eco*RI restriction sites, respectively. The amplified DNA fragments were ligated into the multicloning sited of the pTrcHisA (Invitrogen, Carlstad, USA) previously digested with the same enzymes. The vector pTrcHisA-VcCCHIT1B were transformed into *E.coli* strain TOP10 cells, which were grown at 37 ºC in LB-medium with 50 µg mL-1 ampicillin and 0.8 mM glucose until the optical density at 600 nm reached 0.6 and then induced with 1 mM isopropyl-ß-D-thiogalactoside (IPTG). After 4 h of induction at 37 ºC, cells were harvested by centrifugation at 1760 x *g* for 20 min at 4 ºC and frozen. The purification of VcCHIT1b was carry out by the method described by Kirubakaran et al. (2007), with slight modifications. Cells were disrupted with 1 g of glass beds (150-252 µm, Sigma) in a FastPrep machine (FP120, Bio101, Savant). The protein was properly renatured from inclusion bodies dialyzing gradually against denaturation-renaturation buffer (DR) containing progressively low concentrations of urea (6, 5, 4, 3, 2 and 1 mM) for 24 h each, finally using sterile distilled water. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Protein analysis was performed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean II Cell (Bio-Rad) equipment. The molecular mass of the separated polypeptides was estimated by

comparison to the mobility of pre-stained standard low molecular mass range proteins (Bio-Rad).

After a standard PAGE was performed, proteins were electro-transferred to PVDF membranes (Amersham) with a Mini Trans-Blot Cell (Bio-Rad). The Western blot was probed with a 1:5000 dilution of polyclonal anti-PRQ sera from tobacco, kindly provided by Dr. Fritig (Strasbourg, France) which were detected with a 1:5000 dilution of rabbit antiserum against IgG horseradish peroxidase conjugate (Amersham). The immuno-complexes were visualized using the enhanced chemiluminescence (ECL®) detection system (Amersham).

Recombinant purified VcCHIT1B were tested for antifungal activity against *Botrytis cinerea* (strain provided by the Spanish Type Culture Collection, Universidad de Valencia, Spain) by the hyphal extension-inhibition bioassay. The assay was carried out in 100 mm x 15 mm Petri plates containing 20 mL of potato dextrose agar (PDA). Freshly prepared inoculum (10 µL) was placed in the center and incubated for 24 h at 25 ºC. After the mycelial colony had developed, sterile blank paper disks (0.7 cm in diameter) were placed around and at a distance of 1 cm away from the the mycelial colony. The disk assayed contained sodium acetate buffer 20 mM (control), an aliquot containing 25 µg of purified VcCHIT1b and an aliquot containing 200 µg of bacterial chitinase (*Streptomyces griseus*, Sigma). The plates were incubated for 35 h at 25 ºC in the dark.

The *in vitro* cryoprotective activity was determined following the method described by Romero et al. (2007). Samples, either VcCHTI1b protein fractions or bovine serum albumin (BSA, Sigma, St. Louis, MO, USA), were diluted to varying concentrations and mixed with 2.364 µg of LDH from the stock solution. The resulting solution (300 µL) was frozen three times in liquid nitrogen for 30 s and then thawed at

room temperature for 5 min. LDH enzymatic activity was determined in aliquots of 30 μL of the mixtures in a final volume of 1.5 mL of the reaction assay buffer (80 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM pyruvate and 30 mM NADH) at room temperature. NAD^+ production was monitored as the decrease of absorbance at 340 nm for 4 min. Data is showed as the percentage of the activity present in unfrozen controls.

3. Results and Discussion

In previous work, we have isolated a full-length class I chitinase (*Vcchit1b*) cDNA from the skin of table grape (Romero et al., 2006) and our results indicated that the increase in *Vcchit1b* mRNA levels was paralleled by the change in total decay. To study the effect of low temperature storage and high CO_2 levels on *Vcchit1b* mRNA expression pattern, total mRNA from the skin of CO_2-treated and non-treated grapes stored at 0 ºC was analyzed by northern hybridization. Low temperature increased the levels of the transcript after 3 and 6 days of storage in non-treated grapes. However, the increase in the accumulation of the *Vcchit1b* mRNA in the CO_2-treated grapes was lower and the transcript accumulation was not maintained when treated grapes were transferred to air for 3 days at 0 ºC. In winter rye, transcription of a class I chitinase gene was responsive to cold temperature (Yeh et al., 2000). In this work, we observed that low temperature by itself has a clear effect on *Vcchit1b* gene expression and high CO_2 reduced this response.

To determine whether the *Vcchit1b* cDNA isolated encoded a protein with chitinase activity, and to analyze its functionality, the cDNA fragment corresponding to the mature protein was expressed in TOP10 *E. coli* cells as a pTrcHisA-VcCHIT1b fusion protein. The VcCHIT1b protein was induced in the presence of 1 mM IPTG at 37 ºC for 4 h but we could not purificate the protein because it was aggregated as inclusion

bodies. It is known that recombinant proteins overexpressed in bacteria often form insoluble proteins that contain most of the expression protein (Marston, 1986). Hence, a class I chitinase from barley was produced as insoluble inclusion bodies by Kirubakaran et al. (2007). VcCHIT1b was solubilized and refolded from inclusion bodies by dialysis, and the purified recombinant protein showed a single protein band in SDS-PAGE (Fig. 2, lanes 2), suggesting that the native protein occurs as a monomer. Western blot analysis with an antiserum against tobacco PRQ showed a positive band with the same molecular mass (Fig. 2, lane 3).

We have analyzed the antifungal activity of the recombinant VcCHIT1b using the hyphal growth inhibition assay on agar plates with *B. cinerea* as the test fungus. The assays realized using 25 μg of purified VcCHIT1b showed inhibition of fungal growth (Fig. 3). This inhibition was higher than that observed for a commercial chitinase from *Streptomyces griseus*. These results are agree with the studies showing the ability of classes I, IV and VI chitinases to inhibit growth of fungal hiphae *in vitro* (Derckel et al., 1998) or *in vivo*, in the case of class I chitinases (Benhamou et al., 1993).

We have investigated the ability of the recombinant chitinase to preserve LDH activity following freeze-thaw cycles in comparison with the level of cryoprotection provided by BSA. Without the addition of a cryoprotectant, three freeze-thaw cycles resulted in more than 80% of reduction of LDH activity (Fig. 4). Addition of VcCHIT1b or BSA, in protein:enzyme molar ratios from 0.25 to 1, significantly protected LDH activity. Recombinant VcCHIT1b was more efficient than BSA in protecting LDH at molar ratios from 0.25 to 2.5. At a molar ratio of 1, VcCHIT1b maintained 100% of initial LDH activity. By contrast, a tobacco class I chitinase did not demonstrate any cryoprotective effects, whereas glucanase of class I had potent

cryoprotective activity (Hincha et al., 1997). Liwewise, a PR-5 protein from groundnut showed *in vitro* cryoprotective activity against LDH (Dave and Mitra, 1998).

In conclusion, present investigation report the purification and characterization of a recombinant class I chitinase and the results indicated that VcCHIT1b displays activities as antifungal and potent cryoprotective.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Education and Science (AGL2005-04502/ALI) and Comunidad Autonoma de Madrid (200670M050). I.R. and MT.S.-B. were supported by a postgraduate and postdoctoral I3P contract from the CSIC, respectively. C. F-C. was supported by a predoctoral fellowship from MEC, Spain. The authors are grateful to B. Fritig (Strasbourg, France) for his generosity in providing PR-Q antisera, to Frutaria SAT for providing tables grapes and to Agrotecnica for industrial assistant.

References

Artés-Hernánez, F., Aguayo, E., Artes, F., 2004 Alternative atmosphere treatments for keeping quality of 'Autumn seedless' table grapes during long-term cold storage. Postharvest Biol. Technol. 31, 59-67.

Benhamou, N., Broglie, K., Chet, I., et al., 1993. Cytology of infection of 35s-bean chitinase transgenic canola plants by *Rhizoctonia solani:* cytochemical aspects of chitin breakdown *in vivo*. Plant J. 4 (2), 295-305.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Busam, G., Kassemeyer, H., Matern, U., 1997. Differential expression of chitinases in *Vitis vinifera* L. Responding to systemic acquired resistance activators or fungal challenge. Plant Physiol. 115, 1029-1038.

Collinge, D.B., Kragh, K.M., Mikkelesen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1993. Plant Chitinases. Plant J. 3, 31-40.

Crisosto, C.H., Garner, D., Crisosto, G., 2002. Carbon dioxide-enriched atmospheres during cold storage limit losses from Botrytis but accelerate rachis browning of 'Redglobe' table grapes. Postharvest Biol. Technol. 26, 181-189.

Dave, R.S., Mitra, R.K. A low temperature induced apoplastic protein isolated from *Arachis hypogea*. Phytochem. 4, 2207-2213.

De los Reyes, B.G., Taliaferro, C.M., Anderson, M.P., Melcher, U., McMaugh, S., 2001. Induced expression of the class II chitinase gene during cold acclimation and dehydration of bermudagrass (*Cyndon sp.*) Theoret. Appl. Genet. 103, 297-306.

Derckel, J.P., Audran, J.-C., Haye, B., Lambert, B., Legendre, L., 1998. Characterization induction by wounding and salicylic acid, and activity against *Botrytis cinerea* of chitinases and β-1,3-glucanases of ripening grape berries. Physiol. Plant. 104, 56-64.

Grison, R., Grezesbesset, B., Scheneider, M., Lucante, N., Olsen, L., Legauay, J.J., Toppan, A., 1996. Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nature Biotech. 14, 643-646.

Hincha, D.K., Meins, F.Jr., Schmitt, J.M., 1997. β-1,3-glucanase is cryoprotective *in vitro* and is accumulated in leaves during cold acclimation. Plant Physiol. 114, 1077-1083.

Kirubakaran, S.I., Sakthivel, N., 2007. Cloning and overexpression of antifungal barley chitinase gene in *Escherichia coli*. Protein Expr. Purif 52 (1), 159-166.

Marston, F. A. O., 1986. The purification of eukaryotic polypeptides sysnthesized in *Escherichia coli*. Biochem. J. 240, 1–12.

Robert, N., Roche, K., Lebeau, Y., Breda, C., Boulay, M., Esnault, R., Buffard, D., 2002. Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. Plant Sci. 162, 389–400.

Romero, I., Fernandez-Caballero, C., Goni, O., Escribano, M.I., Merodio, C., Sanchez-Ballesta., M.T., 2007. Molecular characterization of a class I beta-1,3-glucanase from *Vitis vinifera* cv. Cardinal. Plant Sci. (in press).

Romero, I., Sanchez-Ballesta, M.T., Maldonado, R., Escribano, M.I., Merodio, C., 2006. Expression of class I chitinase and β-1,3-glucanase genes and postharvest fungal decay control of table grapes by high CO2 pretreatment. Postharvest Biol. Technol. 41, 9-15.

Sanchez-Ballesta, M.T., Romero, I., Jiménez, J.B., Orea, J.M, González-Ureña, A, Escribano, M.I., Merodio, C., 2007. Involvement of phenylpropanoid pathway in the response of table grapes to low temperature and high CO_2 levels. Postharvest Biol. Technol. 46, 29-35.

Yeh, S., Moffatt, B.A., Griffith, M., Xiong, F., Yang, D.S.C, Wiseman, S.B., Sarhan, F., Danyluk, J., Xue, Y.Q., Hew, C.L., Doherty-Kirby, A., Lajoie, G., 2000. Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiol. 124, 1251-1263.

Figure Legends

Fig. 1. Effect of low temperature and high CO_2 levels on *VcChit1b* mRNA accumulation in the skin of 'Cardinal'. 10 μg of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *VcChit1b* probe. The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining.

Fig. 2. (A) Recombinant VcCHIT1b protein was analysed by SDS-PAGE (12% polyacrylamide) and stained by Coomassie blue. Lanes 2 was loaded with 2 μg of reduced protein. Lane 1 was loaded with low molecular mass reference proteins and sizes are indicated on the left. (B) Immunoblot of a similar gel with reduced VcCHIT1b. The blot was incubated with polyclonal anti-PRQ sera from tobacco.

Fig. 3. Effect of recombinant chitinase from grape and bacterial chitinase on the mycelial growth of *Botrytis cinerea*. Position 1, control (Tris-HCl 50mM); 2, bacterial chitinase (200 μg); 3, Recombinant chitinase (25 μg). Photographs were taken after 48 h incubation.

Fig. 4. Cryoprotection of LDH by recombinant VcCHIT1b. A LDH solution was frozen with different concentrations of VcCHIT1b and BSA. The samples were thawed at room temperature and the LDH activity was measured. The relative activity represents the amount of LDH activity remaining after a freeze-thaw treatment as a percentage of the control enzyme activity. Error bars represent S.E (n=3).

Figure 1

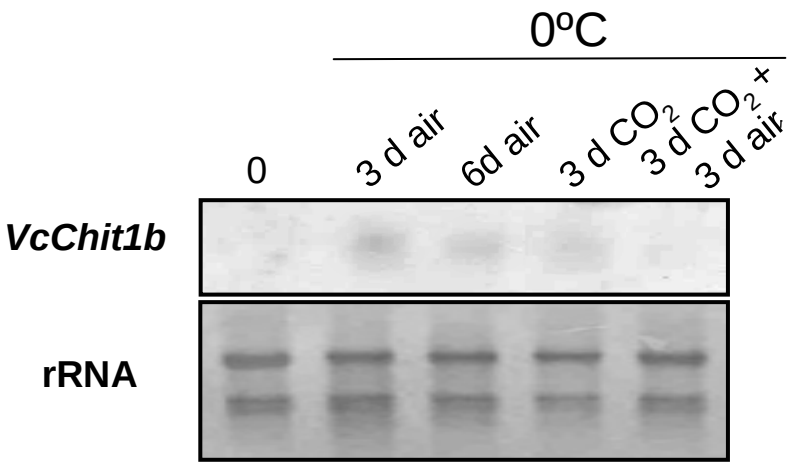


Figure 2

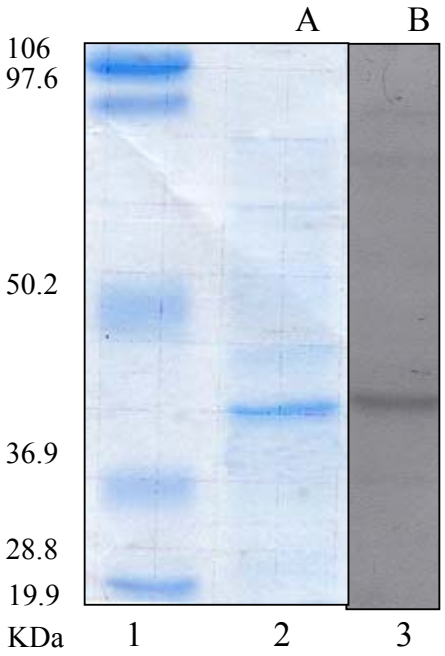


Figure 3

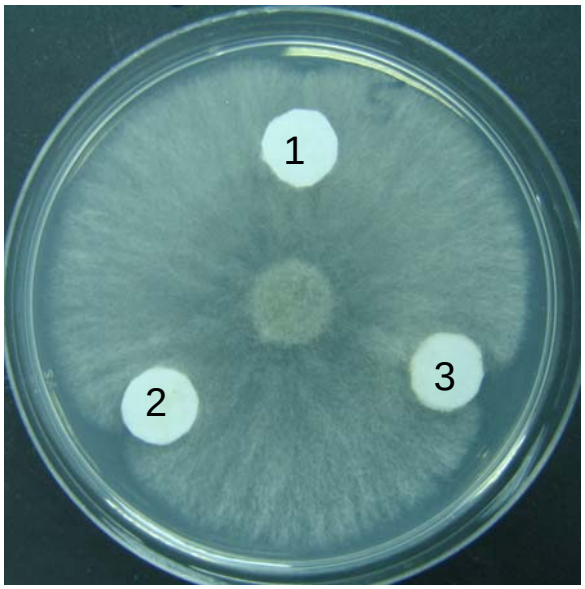
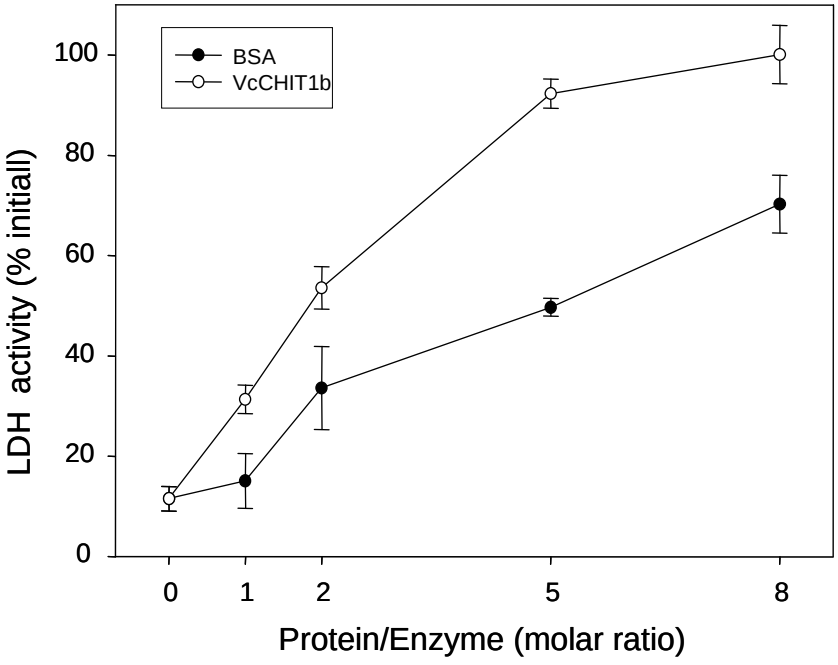


Figure 4


BSA
VcCHIT1b
LDH activity (% initiall)
100
80
60
40
20
0
0
1
2
5
8
Protein/Enzyme (molar ratio)

Discusión

1. Análisis de la infección fúngica de la uva de mesa.

La infección por *Botrytis cinerea* es una de las principales causas de deterioro de uva de mesa durante su conservación postcosecha. Por tanto, uno de los parámetros indicadores de la eficacia de altos niveles de CO_2 y bajas temperaturas en la conservación de uva es el análisis del ataque fúngico. Observamos que la podredumbre aumentó gradualmente en las uvas no tratadas desde el inicio del periodo de conservación a 0ºC. Por el contrario, en las uvas tratadas con CO_2, el ataque por hongos no apareció hasta después de 22 días de almacenamiento. Al final del periodo de conservación, la podredumbre observada en uvas no tratadas fue 5 veces superior a la de uvas tratadas. Resultados similares han sido descrito en uvas de mesa de cosecha temprana Redglobe, donde el desarrollo de *Botrytis* fue significativamente mayor en las bayas almacenadas en aire que en las conservadas mediante tratamientos con CO_2 (Crisosto et al., 2002).

En los ensayos sobre el periodo de vida útil de la uva durante la transferencia a 20ºC tras 12 y 33 días de conservación, se observó que el porcentaje de granos infectados era también menor en los racimos tratados con un 20% de CO_2. Concretamente, cuando la transferencia a 20ºC se realizó pasados 33 días de conservación, el ataque fúngico fue 2,1 veces inferior al observado en frutos no tratados. Por otro lado, cuando la transferencia a 20ºC se realizó tras 12 días a 0ºC, la incidencia del ataque por hongo no presentó cambios significativos en frutos no tratados; mientras que en las uvas tratadas el aumento observado fue de un 30%. También se ha descrito que concentraciones de CO_2 superiores a 10kPa controlaron la incidencia de podredumbres, independientemente de la concentración de O_2, en uva de mesa cv. Redglobe tras el periodo de vida comercial de 3 días (Crisosto et al., 2002).

2. Aislamiento y caracterización del gen que codifica una β-1,3-glucanasa de clase I.

El cDNA *VcGNS1*, que codifica una β-1,3-glucanasa de clase I, se aisló de piel de uva de mesa cv. Cardinal mediante RT-PCR y RACE 5'-3'. La secuencia deducida de aminoácidos de VcGNS1 contiene un residuo de triptófano conservado implicado en la interacción con el sustrato de glucano (Ori et al., 1990), y un residuo de glutamato conservado, que se ha visto que actúa como nucleófilo en el mecanismo catalítico (Varghese et al., 1994). Asimismo, la secuencia deducida de aminoácidos contiene péptidos señal N- y C-terminal de 20 y 21 aminoácidos, respectivamente, presentes en las proteínas β-1,3-glucanasa de clase I. Estas proteínas son sintetizadas como preproteínas y las extensiones N-y C-terminal son eliminadas durante o después del transporte de la proteína a la vacuola (Shinshi et al., 1988).

2.1. Estudio de la expresión de β-1,3-glucanasa de clase I durante la conservación de uva a 0ºC.

En frutos no tratados, el incremento en la acumulación de los niveles de mRNA *VcGNS1* después de 12 días de conservación a 0ºC fue similar al observado al final de la conservación. Por el contrario, para el mismo periodo de tiempo, el incremento en el porcentaje de granos dañados por hongo fue 4 veces superior al final del almacenamiento. En los frutos tratados con CO_2, se observa incluso un descenso en los niveles de acumulación del mRNA *VcGNS1* al tiempo que se detectó un incremento significativo del ataque fúngico. Esta aparente falta de correlación entre los niveles de expresión génica de *VcGNS1* y el ataque fúngico, podría indicar que otros factores distintos de la infección por hongos están involucrados en su inducción. Se ha descrito la inducción de β-1,3-glucanasas en hojas de vid tras la infección por *B. cinerea* (Renault et al., 1996) así como un aumento en los niveles de los transcritos de una glucanasa en hojas de vid infectadas

por *Uncinula necator* y en bayas inmaduras (Jacobs et al., 1999). También se ha descrito la acumulación de glucanasas en respuesta a otros estreses. Así, se ha observado un aumento en la acumulación de mensajeros de una β-1,3-glucanasa de clase II de tomate y de una β-1,3-glucanasa de clase III de mandarina, durante el almacenamiento a bajas temperaturas de estos frutos (Ding et al., 2002; Sanchez-Ballesta et al., 2006).

Con el fin de estudiar el efecto de la conservación a 0ºC en la inducción de *VcGNS1*, se analizó la acumulación de mensajeros al comienzo de la conservación. Se observó que las bajas temperaturas afectaban a la expresión génica de *VcGNS1* independientemente del ataque fúngico. Así, después de 3 días a 0ºC, se observó un gran aumento en los niveles del mRNA *VcGNS1* en la piel de uvas no tratadas, que se mantuvo después de 6 días. Por el contrario, el tratamiento con CO_2 redujo la acumulación del transcrito observada a 0ºC, mientras que se detectó un ligero aumento en los niveles cuando las uvas tratadas fueron transferidas a aire. Asimismo, la actividad glucanasa aumentó en respuesta a las bajas temperaturas, aunque no se observaron diferencias significativas entre frutos tratados y no tratados. Existen numerosos estudios que relacionan la inducción de actividad β-1,3-glucanasa en tejidos vegetativos de plantas en respuesta a estreses abióticos y a la infección por distintos hongos (Fanta et al., 2003; Cota et al., 2007). Lawrence et al. (2000) encontraron niveles de actividad β-1,3-glucanasa superiores en tomates resistentes a la infección fúngica comparados con tomates susceptibles, y estos niveles aumentaron en ambos casos tras la inoculación con el hongo *Alternaria solani*. También se ha descrito un aumento en la actividad β-1,3-glucanasa en respuesta a la infección por *Alternaria alternata* en los cultivares de tomate Sunpride, Jerónimo y Charleston (Cota et al., 2007). Sin embargo, nuestros resultados apuntan a que la β-1,3-glucanasa podría estar implicada en la respuesta de la uva a las bajas temperaturas

independientemente del ataque fúngico. Se han descrito previamente β-1,3-glucanasas ácidas y básicas de centeno que fueron activas frente el frío y que mantuvieron actividad hidrolítica parcial a temperaturas por debajo de 0ºC (Yaish et al., 2006).

2.2. Expresión, purificación y caracterización de la β-1,3-glucanasa de clase I.

Con el objetivo de determinar si el cDNA *VcGNS1* aislado de uva de mesa codifica una proteína con actividad glucanasa, y analizar su funcionalidad *in vitro*, se llevó a cabo la expresión de la proteína en el sistema pTrcHIS en *E. coli*. La proteína recombinante purificada mostró una única banda en geles de SDS-PAGE, y la masa molecular de la proteína recombinante fue de aproximadamente 40 kDa. El aumento observado en la masa molecular de la glucanasa recombinante con respecto al valor teórico (34,6 kDa) es debido a la cola de poli-histidinas que se agregó a la secuencia completa, necesaria para la purificación en columnas de afinidad. La mayoría de las β-1,3-glucanasas caracterizadas pertenecen a una familia de proteínas con una masa molecular de 33-36 kDa, cuya estructura tridimensional, bien sea conformación $(\beta/\alpha)_8$ o estructura en TIM-barril, y actividad enzimática han sido bien establecidas (Varghese et al., 1994; Chen et al., 1995). Sin embargo, existe un número limitado de β-1,3-glucanasas con extensión C-terminal que han sido descritas (Hird et al., 1993; Futamura et al., 2000). Los ensayos de actividad mostraron que la proteína recombinante presenta actividad β-1,3-glucanasa hidrolizando el sustrato laminarina con un pH óptimo de 6, ligeramente superior al determinado en otras proteínas β-1,3-glucanasa (Churngchow et al., 1995; Dicko et al., 2001), pero con una temperatura óptima similar de 45ºC. Cabe destacar que VcGNS1 fue activa en un amplio rango de temperaturas, presentando mayor termoestabilidad que las β-1,3-glucanasas de *Boscia senegalensis* (Dicko et al., 2001) y cebada (Hrmova y Fincher, 1993). Además, la

proteína recombinante presentó a 0ºC un 27% de actividad relativa y la mayor termoestabilidad después de 1 hora de incubación sin sustrato.

Analizando los parámetros cinéticos de la proteína VcGNS1 se observó que la energía de activación E_a fue menor que los valores obtenidos para otras hidrolasas de carbohidratos (Dicko et al., 2001), y constante en el rango de temperaturas estudiado. Es importante destacar que en frutos, no se ha descrito hasta el momento una β-1,3-glucanasa con valores tan bajos de E_a que presente actividad hidrolítica a bajas temperaturas. En cuanto a los parámetros cinéticos, la K_m de la β-1,3-glucanasa fue mayor (0.195 μM) que las descritas para β-1,3-glucanasas de otras plantas (Akiyama et al., 1997; Dicko et al., 2001), mientras que fue del orden de las K_m encontradas en las isoenzimas de β-1,3-glucanasas (GI, GII, y GIII) aisladas de hojas de cebada (Hrmova y Fincher, 1993). Sin embargo, la constante catalítica (K_{cat}) presentó un valor (7.96 s^{-1}) que fue significativamente menor que el de otras glucanasas de plantas (Hrmova y Fincher, 1993; Akiyama et al., 1997). Por otro lado, los resultados indican que VcGNS1 podría tener un comportamiento de endo-hidrolasa ya que no se observó efecto inhibidor de la δ-gluconolactona (potente inhibidor de exoglucanasas) en la actividad enzimática. Este comportamiento se ha observado también en otras glucanasas aisladas (Vogelsang y Barz, 1993; Morohashi y Matsushima, 2000).

2.3. Estudio de la funcionalidad de la β-1,3-glucanasa recombinante.

En cuanto a la funcionalidad de la β-1,3-glucanasa de clase I, se analizó su posible papel como anticongelante y/o crioprotectora. Se han descrito PRs de centeno que actúan como anticongelantes durante la aclimatación al frío (Hon et al., 1995). De hecho, Yaish et al. (2006), expusieron la hipótesis de que glucanasas recombinantes de centeno podrían

actuar como anticongelantes participando en la inhibición de la formación de cristales alargados de hielo, y siendo capaces de degradar glucanos a bajas temperaturas. Sin embargo, nuestros análisis de la actividad anticongelante donde se midió la actividad de histéresis térmica mediante DSC determinaron que VcGNS1 no presentó dicha capacidad. Por otro lado, distintos estudios de la actividad crioprotectora de β-1,3-glucanasas, han mostrado que no es un efecto general de las mismas. Así, en hojas de espinaca y repollo, proteínas β-1,3-glucanasas se acumularon durante la aclimatación al frío, y una glucanasa de clase I tuvo potente actividad crioprotectora (Hincha et al., 1997); mientras que una β-1,3-glucanasa recombinante de clase III de mandarinas sensibles al frío mostró actividad glucanasa pero no crioprotectora (Sanchez-Ballesta et al., 2006). Sin embargo, en este estudio, se ha demostrado que la VcGNS1 tiene actividad crioprotectora frente a la proteína lábil LDH. Los valores de crioprotección observados para VcGNS1 fueron similares a los descritos para la proteína BSA (del inglés *Bovine Serum Albumin*), conocida estabilizadora de proteínas (Tamiya et al., 1985). Se han observado resultados similares en WAP18, una PR10 de mora (Ukaji et al., 2004) y COR15am, una proteína tipo LEA de *Arabidopsis* (Artus et al., 1996) entre otras. Por el contrario, también existen proteínas con actividad crioprotectora que presentan valores superiores a los del BSA. Es el caso de COR85 y PCA60, dehidrinas inducidas por frío con actividades crioprotectoras 4 y 2 veces superiores a las encontradas para el BSA (Kazuoka y Oeda, 1994; Wisniewski et al., 1999). El porcentaje de residuos polares en VcGNS1 es de 40% como ocurre en la proteína WAP18 (Ukaji et al., 2004) y estos autores indican que la baja capacidad crioprotectora encontrada en esta proteína en comparación con otros crioprotectores, puede ser debido a la baja relación de residuos polares en la superficie de la proteína.

3. Aislamiento y caracterización del gen que codifica una quitinasa básica de clase I.

El cDNA *VcCHITt1b* que codifica una quitinasa básica de clase I se aisló de piel de uva de mesa cv. Cardinal mediante RT-PCR y RACE 5'-3'. El análisis de la secuencia de aminoácidos deducida mostró que la proteína VcCHIT1b está compuesta por tres dominios presentes en las quitinasas de clase I: un dominio N-terminal de unión a quitina rico en cisteínas, una región bisagra rica en prolinas y un dominio catalítico altamente conservado. Además, también presenta una extensión C-terminal requerida para la localización vacuolar de la proteína (Di Sansebastiano et al., 1998). El hecho de que la proteína deducida VcCHIT1b presentara una homología del 100% con la quitinasa vacuolar de clase I de hojas de *V.vinifera* cv. Ugni Blanc (Robert et al., 2002) y a la de cultivos celulares de *V.vinifera* cv. Pinot Noir (Busam et al., 1997) hace pensar que las quitinasas de clase I estén muy bien conservadas en las especies de *Vitis*.

3.1. Estudio de la expresión de la quitinasa de clase I durante la conservación de uva a 0ºC.

La acumulación de transcrito *VcCHIT1b* durante la conservación de las bayas a bajas temperaturas fue paralela a la evolución del ataque fúngico que tuvo lugar después de 12 días y que incrementó con el tiempo de almacenamiento. Sin embargo, en las uvas tratadas con CO_2, se detectó un incremento en la expresión génica de la quitinasa de clase I después de 22 días de almacenamiento al tiempo que se observó la aparición de podredumbre en los frutos. Estos resultados muestran una relación entre la acumulación del transcrito *VcCHIT1b* y la evolución de la podredumbre. Se han descrito quitinasas de clase III de calabaza (Métraux et al., 1989) y tabaco (Lawton et al., 1992) que se inducen como respuesta a la infección por patógeno. En cultivos celulares de *V. vinifera* cv. Pinot Noir se observó un aumento en la expresión de una quitinasa de clase III en respuesta a

elicitores fúngicos y bacterianos (Busam et al., 1997). Asimismo, se observó un aumento en la quitinasa básica de clase I aislada de hojas de *V. vinifera* cv. Pinot Noir parcialmente infectadas por *B. cinerea*, siendo los niveles del mensajero más elevados a mayor grado de infección fúngica (Bézier et al., 2002).

En cuanto a la actividad quitinasa, se observaron dos picos de mayor actividad a los 12 y a los 28 días en uvas no tratadas. Sin embargo, en frutos tratados el incremento se observó a los 22 días y fue significativamente menor que los observados en uvas no tratadas. En hojas de vid de cv. Sultana y cv. Cabernet Sauvignon se observó que la actividad quitinasa incrementaba proporcionalmente a la evolución del ataque fúngico de *U. necator* (Jacobs et al., 1999). Asimismo, Salzman et al., (1998) observaron la acumulación de actividad quitinasa en uvas cv Concord, en respuesta a la infección por *B. cinerea*.

El análisis de la expresión génica de *VcCHITt1b* durante la fase inicial del periodo de conservación a bajas temperaturas, mostró que en uvas no tratadas, los niveles del mensajero aumentaban tras 3 y 6 días de almacenamiento a 0ºC. Sin embargo, en uvas tratadas se observó un aumento en los niveles de expresión al finalizar el tratamiento de 3 días, pero no se mantuvo cuando las uvas fueron transferidas a aire. Teniendo en cuenta que nuestros resultados indican que el ataque por hongo comenzó a partir de los 12 días en uvas no tratadas y de los 22 días en uvas tratadas, la acumulación de mRNA de *VcCHITt1b* observada los primeros días de almacenamiento, principalmente en uvas no tratadas, podría ser una respuesta a las condiciones ambientales de conservación. Una proteína de 35 kDa que se acumuló a bajas temperaturas en el apoplasto de hojas de centeno, fue purificada y presentó tanto actividad endoquitinasa como antifúngica, probando que se trataba de una enzima con doble función (Hon et al., 1995).

3.2. Estudio de la expresión de la quitinasa de clase I durante el periodo de vida comercial de uva a 20ºC.

Uvas conservadas a bajas temperaturas experimentan un rápido deterioro, principalmente causado por el ataque de patógenos, cuando son transferidas a temperatura ambiente (Crisosto et al., 2002). De ahí, la importancia de conocer y estudiar los cambios moleculares durante su periodo de vida comercial. Tras el paso a 20ºC después de 33 días de conservación, se observó un aumento brusco en la acumulación de mRNA de *VcCHIT1b* tanto en uvas tratadas como no tratadas, siendo los niveles de expresión similares en ambos casos. También la podredumbre causada por el hongo aumentó tanto en uvas tratadas (3,8 veces) como en no tratadas (1,76 veces) durante el periodo de vida comercial a 20ºC. Sin embargo, en términos globales, el ataque fúngico fue 2,2 veces menor en uvas tratadas que en no tratadas al final de la conservación. Por otro lado, se ha observado un control efectivo contra *B. cinerea* y *Penicillium digitatum* por medio de la combinación de quitinasas mezcladas con otros compuestos antifúngicos (Ali et al., 2003). En este sentido, nuestros resultados han mostrado altos niveles de actividad quitinasa a los 12 días, y tras el paso a 20ºC, principalmente en uvas tratadas, que podría asociarse a la menor incidencia de ataque fúngico observado en las bayas. Sin embargo, durante el periodo de vida útil estudiado al final de la conservación, observamos que mientras en frutos no tratados la actividad quitinasa disminuyó drásticamente alcanzando valores incluso un 45% por debajo de los cuantificados en uvas recién traídas de campo, en las bayas tratadas con CO_2 aumentó alrededor de un 73% con respecto a los valores observados antes del paso a 20ºC. Esta aparente falta de correlación entre los niveles transcripcionales de *VcCHIT1b* y de actividad quitinasa observado durante el periodo de vida comercial tras 33 días de conservación en uvas no tratadas, podría explicarse en términos de cambios en propiedades cinéticas y reguladoras de la enzima quitinasa por

efecto de las bajas temperaturas. Distintos factores, incluyendo modificaciones post-transcripcionales podrían estar implicados en la modulación de la actividad quitinasa.

3.3. Expresión y purificación de una quitinasa de clase I.

La secuencia correspondiente a la proteína quitinasa madura, sin el extremo N-terminal fue expresada en células de *E. coli* TOP 10 como una proteína de fusión pTrcHisA-VcCHIT1b. Durante la inducción de la proteína recombinante se formaron cuerpos de inclusión, por lo se llevo a cabo la solubilización y renaturalización *in vitro* para posteriores análisis. Los cuerpos de inclusión son agregados heterogéneos de elevada densidad, formados por diversas proteínas que poseen cierta estructura secundaria (Carrió et al., 2000). Aunque la expresión de proteínas en la forma de cuerpos de inclusión es a menudo considerada indeseable, su formación puede ser ventajosa, ya que su aislamiento del homogeneizado celular es una manera conveniente y efectiva de purificar la proteína de interés (Surinder y Amulya, 2005). La formación de los cuerpos de inclusión es independiente del huésped empleado para expresar una proteína, ya que se han obtenido incluso cuando se expresa una proteína endógena (expresión homóloga). Tampoco hay una relación directa entre la formación de los cuerpos de inclusión y las características intrínsecas de la proteína, como por ejemplo el peso molecular y la hidrofobicidad. Sólo en el caso de que la proteína forme puentes disulfuro en su estructura, como puede ser el caso de VcCHIT1b con un dominio N-terminal rico en cisteínas, se ha visto cierta relación entre ambos parámetros (Lilie et al., 1998). Hasta el momento sólo se ha descrito la expresión y purificación de una quitinasa recombinante de clase I de cebada que formó cuerpos de inclusión (Kirubakaran et al., 2007). En los últimos años, se han desarrollado métodos novedosos para replegar proteínas con el fin de renaturalizar las proteínas de los cuerpos de inclusión (Tsumoto et al., 2003; Vallejo y Rinas, 2004; Middelberg, 2004). Estos incluyen

tres métodos como son dilución, diálisis o separación en fase sólida (Clark, 1998). Entre los distintos métodos existentes se utilizó la diálisis frente a distintos tampones con concentraciones decrecientes de urea. La quitinasa recombinante purificada mostró una única banda en geles SDS-PAGE. La masa molecular de la quitinasa recombinante se determinó en 34,6 kDa por comparación de su movilidad relativa en geles SDS-PAGE con las de proteínas estándar. Estos resultados se corroboraron mediante Western Blot con antisuero anti PRQ. Se observó un ligero aumento en la masa molecular respecto al valor teórico (31,27 kDa) debido, igualmente, a la cola de poli-histidinas que se agregó a la secuencia completa y que resultaba necesaria para su posterior purificación.

3.4. Estudio de la funcionalidad de la quitinasa recombinante.

La funcionalidad *in vitro* de la quitinasa de clase I también fue estudiada. Se analizó la actividad antifúngica de la VcCHIT1b por inhibición de crecimiento de las hifas de *B. cinerea* sobre placas de agar dextrosa. Utilizando 25 μg de la proteína purificada se observó inhibición del crecimiento del hongo. Esta inhibición fue superior que la observada utilizando quitinasa comercial de *Streptomyces griseus*. Estos resultados están de acuerdo con estudios realizados con quitinasas de clase I, IV y VI en los que se ha observado que estas proteínas son capaces de inhibir el crecimiento de las hifas *in vitro* (Collinge et al., 1993; Derckel et al., 1998) o incluso *in vivo*, en el caso de la quitinasa de clase I (Benhamou et al., 1993). Resultados similares se han observado con endoquitinasas aisladas de granos de maíz, trigo y cebada, que fueron capaces de inhibir la elongación de las hifas del test de hongos (Roberts y Selitrnnikoff, 1988). La combinación de una quitinasa de uva y una osmotina, fue efectiva inhibiendo el crecimiento del micelio de *B. cinerea* (Salzman et al., 1998). Además, la proteína recombinante de quitinasa de clase I de

cebada mostró actividad antifúngica *in vitro* frente a seis hongos fitopatógenos, entre los que también se encontraba *B. cinerea* (Kirubakaran et al., 2007).

Cabe destacar la elevada capacidad crioprotectora de la quitinasa recombinante frente a la proteína LDH utilizando BSA como control. Se ha mostrado que la proteína recombinante VcCHIT1b es más eficiente que el BSA protegiendo la LDH; observándose a la relación molar de 1, que la quitinasa recombinante era capaz de recuperar el 100% de la actividad LDH inicial. Se han descrito distintas proteínas PR inducidas por bajas temperaturas (Tronsmo et al., 1993; Zhu et al., 1993; Gatschet et al., 1993); y algunas de ellas presentan capacidad criprotectora, como es el caso de la PR 10/Bet v1 de mora (Ukaji et al., 2004).

En algunos frutos, se ha observado una relación entre la aclimatación al frío, la adquisición de capacidad crioprotectora y la tolerancia a la congelación (Ukaji et al., 2004; Griffith et al., 1997). Se han descrito quitinasas en centeno inducidas en respuesta a bajas temperaturas y que mostraban capacidad anticongelante (Hon et al., 1995). Asimismo, se han caracterizado dos quitinasas que se acumulan en hojas de centeno durante la aclimatación al frío, que también presentaban actividad anticongelante (Yeh et al., 2000). Sin embargo, la quitinasa recombinante de este estudio, VcCHIT1b, no presentó capacidad anticongelante analizando la actividad de histéresis térmica mediante DSC.

Discusión general

1. Efecto de la aplicación de elevadas concentraciones de CO_2 en los cambios metabólicos asociados con la respuesta de uva de mesa a 0ºC.

La aplicación de un 20% de CO_2 durante 3 días evitó los cambios metabólicos asociados con la respuesta de uva de mesa en la fase inicial de conservación a 0ºC. En la pulpa, estos cambios se centraron principalmente en un aumento de SSC y pH, así como en un descenso de TA en comparación con los valores de los frutos recién traídos de campo. En la piel de la uva, los cambios se concretaron en un aumento significativo tanto del contenido de antocianos totales medido por el método diferencial de pH, como del calculado a partir de la suma de antocianos individuales cuantificados por HPLC-DAD-MS. Se ha descrito previamente, que el contenido de antocianos aumenta en frutos durante la conservación postcosecha a bajas temperaturas (Kalt et al., 1999; Lo Piero et al., 2005; Stiles et al., 2007). El incremento de antocianos se ha intentado correlacionar en distintos estudios con el aumento en la capacidad antioxidante (Wang y Lin, 2000; Jakobek et al., 2007). Sin embargo, debido a que en un mismo extracto puede haber numerosos compuestos antioxidantes con distintos mecanismos de acción y entre los que, además, se pueden establecer reacciones sinérgicas, todavía no existe un consenso sobre cual sería el método más adecuado para efectuar el cálculo de la capacidad antioxidante. Todo ello, unido al elevado número de técnicas empleadas en los trabajos publicados, dificulta la comparación de resultados. En este trabajo se ha empleado el método ABTS, uno de los más empleados en la literatura, para la evaluación de la capacidad antioxidante (Miller et al., 1996; De Beer et al., 2006; Ozgen et al., 2006). Además, considerando que las reacciones que se llevan a cabo para determinar la capacidad antioxidante son fuertemente dependientes del pH (Borkowski et al., 2005; Pérez-Jiménez et al., 2006), la evaluación de la capacidad antioxidante realizada a los extractos se completó con la determinación de la TAC calculada a partir de la suma de la contribución individual de cada antociano. Los

resultados han mostrado un incremento en la capacidad antioxidante de los extractos de piel de uva después de 3 días de conservación a 0ºC paralelo al aumento en la acumulación de cada uno de los antocianos identificadas en esta variedad. El aumento en la capacidad antioxidante de los extractos de uvas no tratadas se debió principalmente a la contribución de la peonidina-3-G, el antociano mayoritario de este cultivar.

El CO_2 evitó el incremento transitorio en el contenido de antocianos totales e individuales. Al finalizar el tratamiento con un 20% de CO_2 la piel de los frutos presentó un valor de la TAC calculada similar a la de los frutos recién cosechados. También se ha descrito en frutos almacenados en CA una menor actividad antioxidante (Van der Sluis 2001; Remón et al., 2004). El estudio detallado del perfil de antocianos en los frutos al finalizar el tratamiento gaseoso permite concluir que los altos niveles de CO_2 evitaron la acumulación de peonidina-3-G y el consiguiente incremento en la capacidad antioxidante. Por el contrario, el tratamiento gaseoso potenció la acumulación de antocianos minoritarios como la pelargonidina-3-G al tiempo que redujo la acumulación de delfinidina-3-G. El descenso significativo en este antociano al finalizar el tratamiento y durante los primeros días de transferencia al aire podría estar relacionado con la mayor pérdida de tonalidad azul-morada observada en los frutos tratados con CO_2, tal y como muestran los valores del ángulo hue; así como con la menor capacidad antioxidante encontrada en estos frutos.

En relación con el metabolismo de los fenilpropanoides, también se ha comprobado que al finalizar el tratamiento gaseoso de 3 días el contenido en *trans*-resveratrol era menor que en los extractos de piel de frutos mantenidos en aire a 0ºC. Más aún, el menor incremento en el contenido de antocianos y el descenso de *trans*-resveratrol observados en uva de mesa cv. Cardinal en respuesta a las elevadas concentraciones de CO_2, se ha relacionado con la menor expresión de genes que codifican enzimas clave en la síntesis de estos compuestos como la PAL, CHS y STS.

La inducción de la expresión de PRs, otra de las respuestas observada frente a las bajas temperaturas, también se vio atenuada en los frutos al finalizar el tratamiento gaseoso. Desde el comienzo del almacenamiento a 0ºC se observó un aumento en los niveles de expresión de los genes de β-1,3-glucanasa y quitinasa, siendo mayor en β-1,3-glucanasas. Dado que existía en la bibliografía información sobre la función de β-1,3-glucanasas y quitinasas de otras plantas como crioprotectoras, anticongelantes (Hincha et al., 1997; Yeh et al., 2000; Ukaji et al., 2004; Yaish et al., 2006) y/o antifúngicas (Hon et al., 1995; Derckel et al., 1998; Kirubakaran et al., 2007), se procedió a estudiar la funcionalidad de las proteínas recombinantes obtenidas por expresión heteróloga. Se observó que las dos proteínas recombinantes purificadas presentaban capacidad crioprotectora; en concreto, la β-1,3-glucanasa presentó una actividad crioprotectora del orden del BSA, mientras que la quitinasa mostró una actividad crioprotectora hasta 2,5 veces superior al BSA en una relación equimolar de proteína/LDH. Sin embargo, ambas proteínas recombinantes no mostraron actividad anticongelante.

2. Efecto residual del CO_2 en la mejora de la uva de mesa, con respecto al ataque por hongo, a través del metabolismo de los fenilpropanoides y PRs.

Durante la transferencia al aire después de 3 días de tratamiento se confirmó el efecto beneficioso de los altos niveles de CO_2 en el mantenimiento de la calidad de la uva de mesa a lo largo de la conservación a 0ºC. Los resultados indican que los racimos tratados con CO_2 presentaron una menor pérdida de peso, así como un menor marchitamiento y oscurecimiento del raquis que los racimos no tratados. Crisosto et al. (2002) describieron signos de marchitamiento del raquis al aplicar altos niveles de CO_2 (15 kPa) dependiendo del estado de desarrollo del fruto. Además, al ser un tratamiento de corta duración, se evitó la aparición de olores y sabores extraños que se pueden producir en condiciones de hipoxia

(Kader, 2002). Los resultados también indican que no se produjeron pardeamientos en la pulpa de las bayas tratadas. Además, los frutos tratados con CO_2 presentaron una significativa menor pérdida de acidez que los no tratados después de 33 días de conservación a 0ºC, lo que explica su menor índice de madurez (SSC/TA).

Fundamentalmente, el empleo de altos niveles de CO_2 redujo y retrasó la aparición de podredumbre en las uvas de mesa. Al final de la conservación a 0ºC, se observó una disminución significativa de los granos infectados por hongos en uvas tratadas con CO_2. Estudiando el posible papel de la ruta de los fenilpropanoides en esta mejora de la calidad de la uva, se observó que los niveles de expresión de *PAL*, *CHS* y *STS* fueron menores en uvas tratadas con CO_2 que en uvas no tratadas. Además, los niveles de antocianos al final de la conservación fueron similares a los de los frutos recién traídos de campo y, tampoco se observaron diferencias significativas en la capacidad antioxidante. Por otro lado, se analizaron los niveles de *trans*-resveratrol en la piel de la uva a lo largo de la conservación. En uva cv. Cardinal el contenido de esta fitoalexina en frutos recién traídos de campo fue de 44,11 μg/g peso fresco. Se ha descrito que el resveratrol se acumula en respuesta a estreses bióticos y abióticos (Liswidowati et al., 1991; Adrian et al., 1998; Cantos et al., 2000). En los ensayos realizados, se observó un incremento significativo en los niveles de *trans*-resveratrol en las uvas no tratadas al final de la conservación coincidiendo con el mayor porcentaje de podredumbre. Por otro lado, el descenso observado en los niveles de *trans*-resveratrol al final del tratamiento gaseoso fue incrementando progresivamente después de la transferencia al aire alcanzando, a los 33 días, valores similares a los de los frutos recién cosechados.

En cuanto a las PRs, se ha descrito el papel de las quitinasas y β-1,3-glucanasas frente al ataque por patógenos (Kim y Hwang, 1994; Salzman et al., 1998). Los resultados muestran una menor acumulación del mRNA de la glucanasa en las uvas tratadas con CO_2

respecto a las no tratadas a lo largo de la conservación. Por el contrario, los niveles de expresión del mRNA de la quitinasa fueron similares en ambos casos aunque se observó un retraso en su inducción en las uvas tratadas. Asimismo, este retraso coincidió con el observado en el desarrollo de la podredumbre. Estudiando la funcionalidad de las dos enzimas, se observó que la β-1,3-glucanasa no presentó, por sí misma, actividad antifúngica frente *B. cinerea* en ensayos *in vitro*. Se ha descrito que la combinación con otras PRs puede tener un efecto sinérgico y potenciar la respuesta antifúngica (Mauch et al., 1988). En este trabajo, se observó un efecto sinérgico cuando la β-1,3-glucanasa se ensayó junto con una quitinasa bacteriana de *S. griseus* (datos no publicados). Sin embargo, la quitinasa sí presentó capacidad antifúngica frente *B. cinerea* a las concentraciones ensayadas.

Conclusiones

1. Los datos obtenidos apuntan a la utilidad comercial del tratamiento de 3 días con altos niveles de CO_2 durante la conservación a 0ºC para mantener la calidad inicial de uva de mesa cv. Cardinal. Así, se observó un mantenimiento de la acidez, una menor pérdida de peso y una disminución del marchitamiento del raquis.

2. Los resultados fisiológicos y moleculares indican que durante la fase inicial de conservación a 0ºC, la uva de mesa induce mecanismos orientados hacia una superación del estrés oxidativo que no se manifiestan con la misma intensidad en uvas tratadas con CO_2 durante 3 días. La protección inducida por el CO_2 se puede observar previamente a la transferencia al aire analizando el contenido total e individual de antocianos.

3. La respuesta a las bajas temperaturas de conservación es transitoria como lo demuestra la recuperación de los valores iniciales de sólidos solubles, antocianos totales, y de los niveles de expresión de los mensajeros de la *PAL* y *CHS*. Este efecto se evita al menos parcialmente con el tratamiento de CO_2, cuyo efecto beneficioso se mantiene hasta el día 22 de la conservación, disminuyendo a partir de este momento. De manera que al final del periodo máximo de conservación no se observan diferencias entre los frutos tratados y no tratados.

4. La protección inducida por el CO_2 frente al ataque fúngico durante la conservación y posterior transferencia a temperatura ambiente no parece estar mediada por el incremento en los niveles de mRNA de la STS, que por otro lado fue concomitante con la acumulación de los niveles de *trans*-resveratrol.

5. La incorporación de un estudio cinético a la evaluación de la capacidad antioxidante mediante el método ABTS de cada uno de los antocianos, unido al estudio de la contribución individual de cada antociano a la capacidad antioxidante total del extracto, proporcionan un conocimiento más completo del efecto de las bajas temperaturas y alto CO_2 en el comportamiento de los antocianos.

6. Se ha identificado por primera vez en piel de uva de mesa, la pelargonidina-3-G, que exhibió una alta capacidad antioxidante y cuya acumulación está bajo control de la temperatura y el CO_2. El tratamiento con CO_2 impidió la acumulación de peonidina-3-G, el antociano mayoritario en uva cv. Cardinal y principal responsable del transitorio incremento de antocianos totales y de la capacidad antioxidante total de uvas durante la fase inicial de conservación a 0ºC.

7. Este estudio demuestra que la fase inicial de conservación a 0ºC de las uvas no tratadas va acompañada de un aumento en los niveles de expresión de los genes que codifican la β-1,3-glucanasa y la quitinasa de clase I. También se observaron aumentos en los niveles de expresión de los transcritos *PAL* y *CHS* como respuesta a las bajas temperaturas, que fueron acompañados de un aumento en el nivel de antocianos y de la capacidad antioxidante.

8. El desajuste metabólico provocado por las bajas temperaturas de conservación se manifestó en un mayor envejecimiento de los tejidos del racimo así como en una mayor y más temprana incidencia de podredumbre. Por el contrario, el tratamiento con CO_2 mantuvo el metabolismo de la baya en condiciones similares a las del fruto recién cosechado permitiendo así el retraso en la aparición de hongos.

9. La efectividad del tratamiento combinado de bajas temperaturas y alta concentración de CO_2 quedaría probada una vez más al prevenir la formación de ROS más que a su inactivación, tal y como se deduce por el patrón de expresión de la *APX*.

10. Los niveles de expresión de la quitinasa y de la β-1,3-glucanasa fueron menores en los frutos tratados con altos niveles de CO_2, coincidiendo con un menor ataque fúngico lo que permite concluir que el efecto beneficioso del CO_2 en el control de la podredumbre no está mediado por la inducción de PRs.

11. Se ha estudiado la funcionalidad de la quitinasa y de la β-1,3-glucanasa de clase I con el fin de conocer la implicación de estas proteínas en la repuesta de la uva de mesa a las bajas temperaturas y al ataque fúngico. La quitinasa recombinante resultó ser hasta 2,5 veces mas crioprotectora que el BSA y también, fue capaz de inhibir el crecimiento de *B. cinerea* en ensayos *in vitro*. Por otro lado, la β-1,3-glucanasa recombinante presentó una capacidad crioprotectora del orden del BSA. Ambas proteínas no mostraron actividad anticongelante.

Bibliografía

Abeles F.B., Bosshart R.P., Forrence L.E., Habig W.H. (1971). Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiology 47: 129-134.

Achuo E.A., Prinsen E., Hofte M. (2006). Influence of drought, salt stress and abscisic acid on the resistance of tomato to *Botrytis cinerea* and *Oidium neolycopersici.* Plant Pathology 55 (2): 178-186.

Adrian M., Jeandet P., Douillet-Breuil A.C., Tesson L., Bessis R. (2000). Stilbene content of mature *Vitis vinifera* berries in response to UV-C elicitation. Journal of Agricultural and Food Chemistry 48(12): 6103-6105.

Adrian M., Jeandet P., Veneau J., Weston L.A., Bessis R. (1997). Biological activity of resveratrol, a stilbenic compound from grapevines, against *Botrytis cinerea*, the causal agent for gray mold. Journal of Chemistry and Ecology 23: 1689-1702.

Adrian M., Rajaei H., Jeandet P., Veneau J., Bessis R. (1998). Resveratrol oxidation in *Botrytis cinerea* conidia. Phytopathology 88: 472-476.

Ahumada M.H., Mitcham E.J., Moore D.G. (1996). Postharvest quality of 'Thompson Seedless' grapes after insecticidal controlled-atmosphere treatments. HortScience 31 (5): 833-836.

Akiyama T., Shibuya N., Hrmova M., et al. (1997). Purification and characterization of a (1-3)-beta-D-glucan endohydrolase from rice (*Oryza sativa*) bran. Carbohydrate Research 297 (4): 365-374.

Aktas L.Y., Guven A. (2007). The appearance of systemic acquired resistance components in SA-treated or naturally infected *Vitis* plants by *Uncinula necator*. Pakistan Journal of Botany 39 (1): 183-191.

Andresen I., Becker W., Schluter K., Burges J., Parthier B., Apel K. (1992). The identification of leaf thionin as one of the main jasmonate induced proteins in barley (*Hordeum vulgare*). Plant Molecular and Biology 19, 193-204.

Antikainen M., Griffith M. (1997). Antifreeze protein accumulation in freezing-tolerant cereals. Physiologia Plantarum 99: 423-432.

Anwar A.K. and Ding S.S. (2004). Molecular cloning, characterization, and expression analysis of two class II chitinases genes from the strawberry plant. Plant Science 166: 753-762.

Apel G., Patterson M.E., Patten K. (1982). The climacteric status of sweet cherry fruit. Hortscience 17 (3): 490-490.

Arai Y., Watanabe S., Kimira M., Shimoi K., Mochizuki R., and Kinae N. (2000). Dietary intakes of flavonols, flavones and isoflavones by Japanase women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. Journal of Nutrition 130:2243-2250.

Archbold D.D., Hamilton-Kemp T.R., Clements A.M., Collins R.W. (1999). Fumigating 'Crimson Seedless' table grapes with (E)-2-hexenal reduces mold during long-term postharvest storage. HortScience 34, 705-707.

Arfaoui A., El Hadrami A., Mabrouk Y., et al. (2007). Treatment of chickpea with *Rhizobium i*solates enhances the expresión of phenylpropanoid defense-related genes in response to infection by *Fusarium oxysporum* sp. Ciceris. Plant Physiology and Biochemistry 45 (6-7): 470-479.

Arnous A., Makris D.P., Kefalas P. (2002). Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece. Journal of Food Composition and Analysis 15 (6): 655-665.

Aronson J.M. (1965). The cell wall. In: Ainsworth G.C., Sussman A.S., eds. The fungi, Vol. 1. London: Academic Press, 49-76.

Artes F., Vallejo F., Martínez J.A. (2001). Quality of brocoli as influenced by film wrapping during shipment. European Food Research and Technology 213 (6): 480-483.

Artes-Hernandez F., Aguayo E., Artes F. (2004). Altenative atmosphere treatments for keeping quality of 'Autumn seedless' table grapes during long cold storage. Postharvest Biology and Technology 31: 59-67.

Artes-Hernandez F., Aguayo E., Artes F. et al. (2007). Enriched ozone atmosphere enhances bioactive phenolics in seedles table grapes alter prolonged shelf life. Journal of the Science of Food and Agriculture 87 (5): 824-831.

Artes-Hernandez F., Artes F., Tomas-Barberan F.A. (2003). Quality and enhancement of bioactive phenolics in Cv. Napoleon table grapes exposed to different postharvest gaseous treatments. Journal of Agricultural and Food Chemistry 51 (18): 5290-5295.

Artes-Hernandez F., Tomas-Barberan F.A., Artes F. (2006). Modified atmosphere packaging preserves quality of SO2-free 'Superior seedless' table grapes. Postharvest Biology and Technology 39 (2): 146-154.

Artus N.N., Uemura M., Steponkus P.L., et al. (1996). Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. Proceedings of the National Academy of Sciences of the United States of America 93 (23): 13404-13409.

Audenaert K., De Meyer G.B., Hofte M.M. (2002). Abscisic acid determines basal susceptibility of tomato *Botrytis cinerea* and suppresses salicylic acid-dependent signalling mechanisms. Plant Physiology 128 (2): 491-501.

Avonce N., Leyman B., Mascorro-Gallardo J.O., Van Dijck P., Thevelein J.M., Iturriaga G. (2004). The Arabidopsis trehalose-6-P synthase AtTPS1 gene is a regulador of glucose, abscisic acid, and stress signaling. Plant Physiology 136: 3649-3659.

Aziz A., Heyraud A., Lambert B. (2004). Oligalacturonide signal transduction, induction of defense-related responses and protection of grapevine against *Botrytis cinerea*. Planta 218 (5): 767-774.

Babu R.M., Sajeena A., Samundeeswari A.V., Sreedhar A., Vidhyasekaran P., Seetharaman K., Reddy M.S. (2003). Induction of systemic resistance to *Xanthomonas oryzae* pv. oryzae by salicylic acid in *Oryza sativa* (L). Journal of Plant Diseases and Protection 110 (5): 419-431.

Baker J., Steele C. and Dure III L. (1988). Sequence and characterization of 6 Lea proteins and their genes from cotton. Plant Molecular Biology 11:277-291.

Balde J.A., Francisco R., Queiroz A., et al. (2006). Immunolocalization of a class III chitinase in two muskmelon cultivars reacting differently to *Fusarium oxysporum* f. sp melonis. Journal of Plant Physiology 163 (1): 19-25.

Ballester A.R., Lafuente M.T., González-Candelas L. (2006). Spatial study of antioxidant enzymes, peroxidase and phenylalanine ammonia-lyase in the citrus fruit- *Penicillium digitatum* interaction. Postharvest Biology and Technology 39: 115-124.

Bartels D., Sunkar R. (2005). Drought and salt tolerance in plants. Critical Review of Plant Science 24: 23-58.

Barth M.M., Weston L.A. and Zhuang H. (1995). Influence of clomazone herbicide on postharvest quality of processing squash and pumpkin. Journal of Agricultural and Food Chemistry 43: 2389-2393.

Beaudry R.M. (1999). Effect of O_2 and CO_2 partial pressure on selected phenomena affecting fruit and vegetable quality. Postharvest Biology and Technology 15: 293-303.

Beck E.H., Fettig S., Knake C., Hartig K., Bhattarai T. (2007). Specific and unspecific responses of plants to cold and drought stress. Journal of Bioscience 32 (3): 501-510.

Bedgoog D.R., Bishop A.G., Prenzler P.D. and Robards K. (2005). Analytical approaches to the determination of simple biophenols in forest trees such as Acer (maple), Betula (birch), Coniferus, Eucalyptus, Juniperus (cedar), Picea (spruce) and Quercus (oak). Analyst 130 (6): 809-823.

Beggs C.J., Kuhn K., Bocker R., et al. (1987). Phytochrome-induced flavonoid biosynthesis in mustard (sinapis-alba l) cotyledons - enzymatic control and differential regulation of anthocyanin and quercetin formation. Planta 172 (1): 121-126.

Ben-Aire R., Sonego L. (1980). Pectolytic enzyme activity involved in woolly breakdown of stored peaches. Phytochemistry 19: 2553-2555.

Benhamou N., Broglie K., Chet I., et al. (1993). Cytology of infection of 35s-bean chitinase transgenic canola plants by *Rhizoctonia solani*: cytochemical aspects of chitin breakdown in-vivo. Plant Journal 4 (2): 295-305.

Benhamou N., Joosten M.H.A.J., De Wit P.J.G.M. (1990). Subcellular localization of chitinase and of potencial substrate in tomato root tissue infected by *Fusarium oxysporium* f sp. Radicis-lycopersici. Plant Physiology 92: 1108-1120.

Beno-Moualem D., Prusky D. (2000). Early events in the development of quiescent infection of avocado fruits against *C. Gloeosporioides*. Phytopathology 90: 553-559.

Bernards M.A., Lewis N.G. (1992). Alkyl ferulates in wound-healing potato-tubers. Phytochemistry 31: 3409-3412.

Berry G., Aked J. (1997). Controlled atmosphere alternatives to the postharvest use of sulfur dioxide to inhibit the development of *Botrytis cinerea* in table grapes, p. 100. In: A.A. Kader (ed.). CA '97 Proc., Vol. 3, Postharvest Horticulture Series No. 17. Postharvest Outreach Program, Univ. of California, Davis.

Bertelli A. A., Giovannini L., Giannessi D., Migliori M., Bernini W., Fregoni M., Bertelli A. (1995). Antiplatelet activity of synthetic and natural resveratrol in red wine. International Journal of Tissue Reactions 17: 1-3.

Bezier A., Lambert B., Baillieul F. (2002). Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. European Journal of Plant Phatology 108:111-120.

Blankenship S.M., Richardson D.G. (1985). Development of ethylene biosynthesis and ethylene-induced ripening in DÁnjou pears during cold requirement for ripening. Journal of American Society Horticultural Science 110:520-523.

Blankenship S.M., Unrath C.R. (1988). Phenylalanine ammonia-lyase and ethylene content during maturation of red and golden delicious apples. Phytochemistry 27: 1001–1003.

Bliffeld M., Mundy J., Potrykus I. (1999). Genetic engineering of wheat for increased resistance to powdery mildew disease. Theoretical and Applied Genetics 98:1079–1086.

Boller T. (1988). Ethylene and the regulation of antifungal hydrolases in plants, Oxf. Surv. Plant Molecular and Cell Biology 5: 145-174.

Borkowski T., Szymusiak H., Gliszczynska-Swiglo A., et al. (2005). Radical scavenging capacity of wine anthocyanins is strongly pH-dependent. Journal of Agricultural and Food Chemistry 53 (14): 5526-5534.

Boss P.K., Davies C., Robinson S.P. (1996). Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiology 111: 1059-1066.

Bouchereau A., Aziz A., Larher F., Martin-Tanguy J. (1999). Polyamines and environmental challenges: Recent Development. Plant Science 140: 103125.

Bowen P., Menzies J., Ehret D., Samuels L., Glass A.D.M. (1992). Soluble silicon sprays inhibit powdery mildew development on grape leaves. Journal of the American Society of Horticultural Science 17: 906–912.

Bowler C., Vancamp W., Vanmontagu M., Inze D. (1994). Superoxide dismutase in plants [Review]. Critical Reviews in Plant Sciences 13: 199-218.

Bowles D.J. (1990). Defense-related proteins in higher plants. Annual Review of Biochemistry 59: 873-907.

Bravo L.A., Gallardo J., Navarrete A., Olave N., Martínez J., Alberdi M., Close T.J., Corcuera L.J. (2003). Cryoprotective activity of a cold induced dehydrin purified from barley. Physiologia Plantarum 118: 262–269.

Bray E.A. (1993). Molecular responses to water-deficit. Plant Physiology 103: 1035-1040.

Brehm I., Preisig-Müller R., Kindl H. (1999). Grapevine protoplasts as a transient expression system for comparison of stilbene synthase genes containing cGMP-responsive promoter elements. Z. Naturforsch. (C) 54: 220-229.

Breuil A.C., Adrian M., Pirio N., et al. (1998). Metabolism of stilbene phytoalexins by Botrytis cinerea: 1. Characterization of a resveratrol dehydrodimer. Tetrahedron Letters 39 (7): 537-540.

Brison L.F., Tenhaken R., Lamb C. (1994). Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell 6: 1703-1712.

Broglie K., Chet I., Holliday M., Cressman R., Biddle P., Knowlton S., Mauvais C.J., Broglie R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254: 1194-1197.

Broglie K.E., Biddle P., Cressman R., Broglie R. (1989). Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. Plant Cell 1: 599-607.

Broglie K.E., Gaynor J.J., Broglie R.M. (1986). Ethylene-regulated gene expression: molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proceedings of the National Academy of Science in U.S.A. 18: 6820–6824.

Brouillard R. (1982). Chemical Structure of anthocyanins. In: Anthocyanins as food colors (P.1 ed.), Academic Press, New York, pp.1-38.

Brouillard R. (1983). The *in vivo* expression of anthocyanin colour in plants. Phytochemistry 22: 1311–1365.

Browse J., Xin Z. (2001). Temperature sensing and cold acclimation. Crrent Opinion in Plant Biology 4: 241-246.

Brunner F., Stintzi A., Fritig B., Legrand M. (1998). Substrate specificities of tobacco chitinases. Plant Journal 14: 225-234.

Bucciaglia P.A., Smith A.G. (1994). Cloning and characterization of Tag1, a tobacco anther β-1,3-glucanase expressed during tetrad dissolution. Plant Molecular Biology 24: 903-914.

Busam G., Kassemeyer H., Matern U. (1997). Differential expression of chitinases in *Vitis vinifera* L. Responding to systemic acquired resistance activators or fungal challenge. Plant Physiology 115: 1029-1038.

Cantos E., Espín J.C., Tomás-Barberán F.A. (2002). Varietal Differences among the Polyphenol Profiles of seven Table Grapes Cultivars Studied by LC-DAD-MS-MS. Journal of Agriculture and Food Chemistry 50: 5691-5696.

Cantos E., García-Viguera C., Pascual-Teresa S., Tomas-Barberan F.A. (2000). Effects of postharvest ultraviolet irradiation on resveratrol and other phenolics of cv. Napoleon table grapes. Journal of Agricultural and Food Chemistry 48: 4606-4612.

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell. 1997 88(1):57–63.

Cao, H., Bowling, S.A., Gordon, A.S., Dong, X. (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell. 1994 6(11): 1583–1592.

Carreño J., Almela L., Martínez A., Fernández-López J.A. (1997). Chemotaxonomical classification of red table grapes based on anthocyanin profile and external colour. Lebensmittel-Wissenschaft und Technologie 30: 259-265.

Carreño J., Martínez A. (1995). Proposal of an index for the objetive evaluation of the colour of red table grapes. Food Research International 28: 373-377.

Carrió M.M., Cubarsi R., Villaverde A.(2000). Fine architecture of bacterial inclusion bodies. FEBS Letters 471(1):7-11.

Castresana, C., de Carvalho, F., Gheysen, G., Habets, M., Inze, D., Van Montagu, M., (1990). Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* beta-1,3-glucanase gene. Plant Cell 2: 1131-1143.

Cervone F., Hahn M.G., Delorenzo G., et al. (1989). Host-Pathogen interactions.33. A Plant Protein Converts a Fungal Pathogenesis Factor into an Elicitor of Plant Defense Responses. Plant Physiology 90(2):542-548.

Cessna S.G., Sears V.E., Dickman M.B., Low P.S. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. Plant Cell 12 (11): 2191-2199.

Chalker-Scott L., Fuchigami L.H., Harber R.M. (1989). Spectrophotometric measurement of leached phenoli-compounds as an indicator of freeze damage. Journal of the American Society for Horticultural Science 114: 315-319.

Chalutz (1973). Ethylene-induced phenylalanine ammonia-lyase activity in carrot roots. Plant Physiology 51:1033–1036.

Chang M.M., Hadwiger L.A., Horovitz D. (1992). Molecular characterization of a pea β-1,3-glucanase induced by Fusarium solani and chitosan challenge. Plant Molecular Biology 20: 609-618.

Chardonnet C., L'Hyvernay A.L., Doneche B. (1997). Effect of calcium treatment prior to *Botrytis cinerea* infection on the changes in pectic composition of grape berry. Physiological and Molecular Plant Pathology 50: 213–218.

Chen J-Y., Wen P-F, Kong W-F., Pan Q-H., Zhan J-C., Li J-M., Wan S-B., Huang W-D. (2006). Effect of salicylic on phenylpropanoids and phenylalanine ammonia-lyase in harvested grape berries. Postharvest Biology and Technology 40: 64-72.

Chen L., Fincher G.B., Hoj P.B. (1993). Evolution of polysaccharide hydrolase substrate specificity. Catalytic amino acids are conserved in barley 1,3-1,4- and β-1,3-glucanases. Journal of Biology and Chemistry 268: 13318-13326.

Chen L., Garret T.P.J., Fincher G.B., Hoj P.B. (1995). A tetrad of ionisable amino acids is important for catalysis in barley β-1,3-glucanases. Journal of Biology and Chemistry 270: 8093-8101.

Chervin C., El-Kereamy A., Roustan J.P., Latche A., Lamon J., Bouzayen M. (2004). Ethylene seems required for the berry development and ripening in grape, a non-climacteric fruit. Plant Science 167 (6): 1301-1305.

Christie P.J., Alfenito M.R., Walbot V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta 194: 541-549.

Chu C.L., Liu W.T., Zhou T. (2001). Fumigation of sweet cherries with tymol and acetic acid to reduce post harvest brown rot and blue mold rot. Fruits 56: 123-130.

Churngchow N., Suntaro A., Wititsuwannnakul R. (1995). Beta-1,3-glucanase isozymes from the latex of hevea-brasiliensis. Phytochemistry 39 (3): 505-509.

Clark E.D. (1998). Refolding of recombinant proteins. Current Opinion in Biotechnology 9: 157-163.

Close T.J. (1997). Dehydrins: A commonalty in the response of plants to dehydration and low temperature. Physiologia Plantarum 100: 291-296.

Clydesdale F.M. (1993). Color as a factor in food choice. Critical Reviews in Food Science and Nutrition 33 (1): 83-101.

Colditz F., Niehaus K., Krajinski F. (2007). Silencing of PR-10-like proteins in *Medicago truncatula* results in an antagonistic induction of other PR proteins and in an increased tolerance upon infection with the oomycete *Aphanomyces euteiches.* Planta 20: 59-71.

Collinge D.B., Kragh K.M., Mikkelesen J.D., Nielsen K.K. Rasmussen U., Vad K. (1993). Plant Chitinases. Plant Journal 3: 31-40.

Cota[a] I.E., Troncoso-Rojas[a] R., Sotelo-Mundo[b] R., Sánchez-Estrada[a] A., Tiznado-Hernández[a] M.E. (2007). Chitinase and β-1,3-glucanase enzymatic activities in response to infection by *Alternaria alternata* evaluated in two stages of development in different tomato fruit varieties. Scientia Horticulturae 112 (1): 42-50.

Cramer C.L., Edwards K., Dron M., Liang X., Dildine S.L., Bolwell G.P., Dixon R.A., lamb C.J., Schuch W. (1989). Phenylalanine ammonia-lyase gene organization and structure. Plant Molecular Biology 12:367-383

Creasy L.L., Coffee M. (1988). Phytoalexin production potential of grape berries. Journal of American Society of Horticultural Science 113: 230–234.

Crisosto C.H., Garner D., Crisosto G. (2002)(b). High carbon dioxide atmospheres affect stored 'Thompson seedless' table grapes. Hortscience 37 (7): 1074-1078.

Crisosto C.H., Garner D., Crisosto G. (2002). Carbon dioxide-enriched atmospheres during cold storage limit losses from *Botrytis* but accelerate rachis browning of 'Redglobe' table grapes. Postharvest Biology and Technology 26:181-189.

Crisosto C.H., Mitchell F.G. (2002). Postharvest handling systems: table grapes. In: A.A. Kader, Editor, Postharvest Technology of Horticultural Crops, Publication 3311, University of California, pp. 357–363.

Crisosto C.H., Smilanick J.L., Dokoozlian N.K., Luvisi D.A. (1994). Maintaining table grape postharvest quality for long distant markets. In: Proceedings of the International Symposium on the Table Grape Production. Anaheim, California, pp. 195-199.

Dai G.H., Andary C., Mondolot-Cosson L., Boubals D. (1995). Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera*, *V. rupestris* and *V. rotundifolia* and the downy mildew fungus, *Plasmopora viticola*. Physiology and Molecular Plant Pahology 46:177-188.

Daie J., Campbell W.F., Seeley S.D. (1981). Temperature-stress induced production of abcisic acid and dihydrophaseic acid in warm and cool season crops. Journal of American Society Horticultural Science 106:11-13.

Dangyang K., Salveit M.E. (1989). Wound-induced ethylene production, phenolic methabolism and susceptibility to russet spotting in iceberg lettuce. Physiologia Plantarum 76:412-418.

Darras A.I., Joyce D.C., Terry L.A., et al. (2006). Postharvest infection of *Freesia hybrida* flowers by *Botrytis cinerea*. Australasian Plant Pathology 35 (1): 55-63.

Das E., Gürakan G.C., Bayindirli A. (2006). Effect of controlled atmosphere storage, modified atmosphere packaging and gaseous ozone treatment on the survival of *Salmonella* Enteritidis on cherry tomatoes. Food Microbiology 23: 430-438.

Dat J.K., Foyer C.H., Scott I.M. (1998)(a). Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. Plant Physiology 118 (4): 1455-1461.

Dat J.K., Lopez-Delgado H., Foyer C.H., Scout I.M. (1998)(b). Parallel changes in H2O2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiology 116(4): 1351-1357.

Dave R.S., Mitra R.K. (1998). A low temperature induced apoplastic protein isolated from *Arachis hypogea*. Phytochemistry 4: 2207-2213.

Davies C., Robinson S.P. (2000). Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening. Cloning and characterization of cDNAs encoding putative cell wall and stress response proteins. Plant Physiology 122 (3): 803-812.

De Beer D., Joubert E., Marais J., Manley M. (2006). Unravelling the Total Antioxidant Capacity of Pinotage Wines : Contribution of phenolic compounds. Journal of Agricultural and Food Chemistry 54: 2897-2905.

De los Reyes B.G., Taliaferro C.M., Anderson M.P., Melcher U., McMaugh S. (2001) Induced expression of the class II chitinase gene during cold acclimation and dehydration of bermudagrass (*Cyndon sp.*) Theoretical and Applied Genetics 103: 297-306.

De Vries-Paterson R.M., Jones A.L., Cameron A.C. (1991). Fungistatic effects of carbon dioxide in a package environment on the decay of Michigan sweet cherries by *Monilinia fructicola*. Plant Disease 75: 943–946.

Deighton N., Brennan R., Finn C., et al. (2000). Antioxidant properties of domesticated and wild Rubus species. Journal of the Science of Food and Agriculture 80 (9): 1307-1313.

Del Cura B., Escribano M.I., Zamorano J.P., Merodio C. (1996). High carbon dioxide delays postharvest changes in RuBPCase and polygalacturonase-related protein in cherimoya peel. Journal of American Society of Horticulturae Science 121: 735–739.

Dempsey D.A., Shah J., Klessig D.F. (1999). Salycilic acid and disease resistance in plants. Critical Reviews in Plant Science 18: 547-575.

Deng Y., Wu Y., Li Y. (2007). Effects of high CO2 and low O2 atmospheres on the the berry drop of 'Kyoho' grapes. Food Chemistry 100: 768-773.

Deng Y., Wu Y., Li Y.F., Yang M.D., Shi C.B., Zheng C.J. (2006). Effects of high O_2 pretreatment and gibberellic acid on sensorial quality and storability of table grapes. Food Science and Technology International 12 (4): 307-313.

Derckel J.P., Audran J.-C., Haye B., Lambert B., Legendre L. (1998). Characterization induction by wounding and salicylic acid, and activity against *Botrytis cinerea* of chitinases and β-1,3-glucanases of ripening grape berries. Physiologia Plantarum 104: 56-64.

Derckel J.P., Baillieul F., Manteau S., Audran J.C., Haye B., Lambert B., Legendre L. (1999). Differential induction of grapevine defenses by two strains of *Botrytis cinerea*. Phytopathology 89: 197-203.

Dercks W., Creasy L.L. (1989). The significance of stilbene phytoalexins in the *Plasmopara viticola*-grapevine interaction. Physiological and Molecular Plant Pathology 34:189–202.

Dewick P.M. (1994). The biosynthesis of shikimate metabolites. Natural Product Reports 11: 173-203.

Di Sansebastiano G.P., Paris N., Marc-Martin S., Neuhaus J.M. (1998). Specific accumulation of GFP in a non-acidic vacuolar compartment via a C-terminal propeptide-mediated sorting pathway. Plant Journal 15: 449-457.

Díaz-Perales A., Colladsa C., Blanco C., et al. (1998). Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chesnut and avocado allergens: Journal of Allergy Clinical Immunology 102:127-33.

Dicko M.H., Searle-van Leeuwen M.J.F., Hilhorst R., Traore A.S., Beldman G. (2001). Polysaccharide hydrolases from the leaves of *Boscia senegalensis:* properties of the endo-(1→3)-ß-D-glucanase. Applied Biochemistry and Biotechnology 94: 225-241.

Ding C.K., Wang C.Y., Gross K.C., Smith D.L. (2002). Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. Planta. 214: 895-901.

Dixon R.A. (2001). Natural products and plant disease resistance. Nature 411: 843-847.

Dixon RA, Pavia NL. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085-97.

Dokoozlian N., Luvisi D., Moriyama M., Schrader P. (1995). Cultural practices improve color, size of 'Crimson Seedless'. California Agriculture 49:36–40.

Dong Y.H., Beuning L., Davies K., et al. (1998). Expression of pigmentation genes and photo-regulation of anthocyanin biosynthesis in developing Royal Gala apple flowers Australian Journal of Plant Physiology 25 (2): 245-252.

Dopico B, Lowe AL, Wilson ID, Merodio C, Grierson D. (1993). Cloning and characterization of avocado fruit messenger-RNAs and their expression during ripening and low-temperature storage. Plant Molecular Biology 21 (3): 437-449.

Dore I., Legrand M., Cornelissen B.J.C., Bol J.F. (1991). Subcellular localization of acidic and basic PR proteins in tobacco mosaic virus-infected tobacco. Archives of Virology 120: 97-107.

Drolet G., Dumbroff E.B., Legge R.L., Thompsom J.E. (1986). Radical scavenging properties of polyamines. Phytochemistry 25:367-371.

Elad Y. (1997). Responses of plants to infection by *Botrytis cinerea* and novel means involved in reducing their susceptibility to infection. Biological Reviews 72: 381-422.

Ernst D., Schraudner M., Langebartels C., Sandermann Jr. H. (1992). Ozone-induced changes of mRNA levels of β-1,3-glucanases, chitinases and 'pathogenesis-related' protein 1b in tobacco plants. Plant Molecular Biology 20: 673-682.

Escribano M.I. , Merodio C. (2000). Poliaminas en la fisiología de la postrecolección: una clave en el proceso de maduración y mejora de la conservación de frutos. A.J. Matilla (ed.). Fisiología Hormonal, pp. 233-242.

Escribano M.I., Aguado P., Reguera R.M., Merodio C. (1997). The effect of high carbon dioxide at low temperature on ribulose 1,5- biphosphate carboxylase abd polygalacturonase protein levels in cherimoya fruit. Journal of American Society for Horticultural Science 122, 258-262.

Eshel D., Miyara I., Ailing T., et al. (2002). pH regulates endoglucanase expression and virulence of *Alternaria alternata* persimmon fruit. Molecular Plant-Microbe Interactions 15 (8): 774-779.

Fallik E., Archbold D.D., Hamilton-Kemp T.R., Clements A.M., Collins R.W., BArth M.E. (1998). (E)-2-Hexenal can stimulate Botrytis cinerea growth in vitro and on strawberry fruit in vivo during storage. Journal of American Society in Horticultural Science 123, 875-881.

Fanta N., Ortega X., Perez L.M. (2003). The development of Alternaria alternata is prevented by chitinases and beta-1,3-glucanases from *Citrus limon* seedlings. Biological Research 36 (3-4): 411-420.

Faragher J.D. (1983). Temperature regulation of anthocyanin accumulation in apple skin. Journal of Experimental Botany 34**:** 1291–1298.

Faragher J.D., Chalmers D.J. (1977). Regulation of anthocyanin synthesis in apple skin .3. Involvement of phenylalanine ammonia-lyase. Australian Journal of Plant Physiology 4:133-141.

Farber J.M. (1991). Microbiological aspects of modified atmosphere packaging-a review. Journal of Food Protection 54: 58-70.

Félix G., Meins F. Jr (1986). Developmental and hormonal regulation of β-1,3-glucanase in tobacco. Planta 167: 206-211.

Ferrari S., Plotnikova J.M., De Lorenzo G., Ausubel F.M. (2003). *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2 but not SID2, EDS5 or PAD4. The Plant Journal 35: 193-205.

Feys B.J., Parker J.E., (2000). Interplay of signaling pathways in plant disease resistance. Trends in Genetics 16: 449–455.

Flower D.J., Lidlow M.M. (1986). Contribution of osmotic adjustment to the dehydration tolerance of water stressed pigeon pea (*Cajanas cajan (L)* Millsp) leaves, Plant Cell Environment 9: 33–40.

Forsyth J.L., Shewry P.R. (2002). Characterization of the major proteins of tubers of yam bean (*Pachyrhizus ahipa*). Journal of Agricultural and Food Chemistry 50 (7): 1939-1944.

Fridman E., Pichersky E. (2005). Metabolomics, genomics, proteomics and the identification of enzymes and their substrates and products. Current Opinion in Plant Biology 8: 242-248.

Fritz C., Palacios-Rojas N., Feil R., et al. (2006). Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. Plant Journal 46 (4): 533-548.

Fujisawa H., Seto H., Yoshida S., Kamuro Y. (1996). Promoting effects of jasmonic acid analog, *n*-propyl dihydrojasmonate (PDJ) on plant growth. Proceedings of 23rd Annual Meeting Plant Growth Regulation of American Society, pp. 111-116.

Fulcher R.G., Mc Cully, M.E., Setterfield, G., Sutherland, J. (1976). β-1, 3-Glucans may be associated with cell plate formation during cytokinesis. Canadian Journal of Botany 54, 459–542.

Fung R.W.M., Wang C.Y., Smith D.L., Gross K.C., Tian M.S. (2004). MeSa and MeJa increase steady-state transcript levels of alternative oxidase and resistance against chilling injury in sweet peppers (*Capsicum annuum* L.) Plant Science 166: 711-719.

Futamura N., Mori H., Kouchi H., Shinohara K. (2000). Male flower-specific expression of genes for polygalacturonase, pectin methylesterase and β-1,3-glucanase in a dioecious willow (*Salix gilgiana* Seemen). Plant Cell physiology 41: 16-26.

Gabler F.M., Smilanick J.L. (2001). Postharvest control of table grape gray mold on detectable berries with carbonate and bicarbonate salts and disinfectants. American Journal of Enology and Viticulture 52: 12-20.

Garcia-Alonso M., Rimbach G., Rivas-Gonzalo J.C. et al. (2004). Antioxidant and cellular activities of anthocyanins and their corresponding vitisins A—studies in platelets, monocytes, and human endothelial cells. Journal of Agricultural and Food Chemistry 52:3378–3384.

Gatschet M.J., Taliaferro C.M., Anderson J.A., Porter D.R, Anderson M.A. (1993). Cold acclimation and alterations in protein synthesis by bermudagrass crowns. Journal of American Society Horticulturae Science 119: 477-480.

Geny L., Broquedis M., MartinTaguy J., Bouard J. (1997). Free, conjugated, and wall-bound polyamines in various organs of fruiting cuttings of *Vitis vinifera* L. cv. Cabernet Sauvignon. American Journal of Enology and Viticulture 48 (1): 80-84.

Gil M.I., Conesa M.A., Artés F. (1999). Modified atmosphere packaging of fresh-cut tomato. Acta Horticulturae 553.

Gil M.I., Holcroft D.M., Kader A.A. (1997). Changes in strawberry anthocyanins and other polyphenols in response to carbon dioxide treatments. Journal of Agricultural and Food Chemistry 45: 1662-1667.

Gil-Izquierdo A.,Gil M.I., Ferreres F., Thomás-Barberán F.A. (2001). In vitro availability of flavonoids and other phenolics in orange juice. Journal of Agricultural and Food Chemistry 49: 1035–1041.

Gindro K., Pezet R. (2001).Effects of long-term storage at different temperatures on conidia of *Botrytis cinerea* Pers. FEMS Microbiology letters 204(1): 101-104.

Girbau T, Stummer B.E., Pocock K.F., Baldock G.A., Scott E.S., Waters E.J. (2004). The effect of *Uncinula necator* (powdery mildew) and *Botrytis cinerea* infection of grapes on the levels of haze-forming pathogenesis-related proteins in grape juice and wine. Australian Journal of Grape and Wine Research 10 (2): 125-133

Glazebrook, J. (2001) Genes controlling expression of defense responses in Arabidopsis- status. *Curr.Opin.Plant.Biol.* 4: 301-308

Goto-Yamamoto, N.;Wan, G. H.;Masaki, K.;Kobayashi, S. 2002 Structure and transcription of three chalcone synthase genes of grapevine (*Vitis vinifera*) Plant Science 162 (6): 867-872.

Grace S.C., Logan B.A. (2000). Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 355: 1499-1510.

Graham D., Patterson B.D. (1982). Responses of plants to low, non freezing temperatures: proteins, metabolism and acclimation. Annual Review of Plant Physiology 33: 347-372.

Graham L.S., Sticklen M.B. (1994). Plant Chitinases. Canadian Journal of Botany 72: 1057-1083.

Grant J.J., Loake G. J. (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiology 124: 21-29.

Grayer R. J., Harborne J. J. (1994). A survey of antifungal compounds from higher plants 1982-1993. Phytochemistry 37:19-42.

Grierson W., Vines H.M., Oberbach M.F., et al. (1966). Controlled atmosphere storage of Florida and California lemons. Proceedings of the American Society for Horticultural Science 88: 311.

Griffith M., Antikainen M., Hon W.C., et al. (1997). Antifreeze proteins in winter rye Physiologia Plantarum 100 (2): 327-332.

Griffith M., Yaish M.F.W. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. Trends in Plant Science 9: 399-405.

Grimmig B., Gonzalez-Perez M.N., Welzl G., Penuelas J., Schubert R., Hain R., Heidenreich B., Betz C., Langebartels C., Ernst D., Sandermann Jr. H. (2002). Plant Physiology 40: 865-870.

Grimplet J., Deluc L.G., Tillett R.L., et al. (2007). Tissue-specific mRNA expression profiling in grape berry tissues. BMC Genomics 8: 187.

Grison R., Grezesbesset B., Scheneider M., Lucante N., Olsen L., Legauay J.J., Toppan A. (1996). Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nature Biotechnology 14: 643-646.

Grover A., Ashpal Y., Arnab S., Nidhi A., Kirti P.B., Sherma R.P. (2001) Removal of vacuolar targeting signal from class I vacuolar chitinase leads to its extracellular secretion in transgenic tobacco. Journal Plant Biochemistry and Biotechnology 10: 139-142.

Guelfat-Reich S., Safran B. (1971). Indices of Maturity for Table Grapes as Determined by Variety. American Journal of Enology and Viticulture 22: 13-18.

Gunata Y.Z., Bayonove C.L., Baumes R.L., et al., (1985)(a). The aroma of grapes –Localization and evolution of free and bound fractions of some grape aroma components cv. Muscat during 1st development and maduration. Journal of the science of food and agriculture 36 (9): 857-862.

Gunata Y.Z., Bayonove C.L., Baumers R.L., et al. (1985)(b). The aroma of grapes.1. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. Journal of Chromatography 331 (1): 83-90.

Gunata Y.Z., Bayonove C.L., Baumes R.L., et al. (1986). Changes in free and bound fractions of aromatic components in vine leaves during development of muscat grapes.3. Phytochemistry 25(4): 943-946.

Gundlach H., Muller M.J., Kutchan T.M., et al. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant-cell cultures. Proceedings of the National Academy of Sciences of the United States of America 89 (6): 2389-2393.

Gupta A.S., Webb R.P., Holaday A.S. Allen –R.D. (1993). Overexpression of superoxide dismutase protects plants from oxidative stress: induction of ascorbate peroxidase in superoxide dismutase-overexpression plants. Plant Physiology 103:1067-1073.

Haake V., Cook D., Riechmann J.L. et al. (2002). Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. Plant Physiology 130 (2): 639-648.

Hadas Y., Goldberg I., Pines O., et al. (2007). Involvement of gluconic acid and glucose oxidase in the pathogenicity of *Penicillium expansum* in apples. Phytopathology 97 (3): 384-390.

Haga M., Haruyama T., Kano H., Sekisawa Y., Usushizaki S. and Matsumoto K. (1988). Dependence on ethylene of the induction of phenylalanine ammonia-lyase activity in rice leaf infected with blast fungus. Agricultural and Biological Chemistry 2: 943-950.

Hahlbrock, K., Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. Annual Review of Plant Physiology and Plant Molecular Biology 17: 425-429.

Hakkinen S.H., Karenlampi S.O., Mykkanen H.M., et al. (2000). Influence of domestic processing and storage on flavonol contents in berries. Journal of Agricultural and Food Chemistry 48 (7): 2960-2965.

Hara M., Terashima S., Kuboi T. (2001). Characterization and cryoprotective activity of cold-responsive dehydrin from *Citrus unshiu*. Journal of Plant Physiology 158 (10): 1333-1339.

Harbone J. B., Grayer R. J. (1988). The anthocyanins. In the Flavonoids: Advances in Research since 1980; Harbone, J. B., Ed.; Chapman and Hall: New York, 1988.pp.1-20.

Harbone J.B. (1967). Comparative Biochemistry of the Flavonoids. New York, NY: Academic Press. pp. 1–30.

Harbone, J. B. (1994). The flavonoids. Advances in Research Since 1986. Chapman and Hall, London. p. 676.

Hart C.M., Nagy F., Meins F.Jr. (1993). A 61 bp enhancer element of the tobacco β-1,3-glucanase B gene interacts with one or more regulated nuclear proteins. Plant Molecular Biology 21: 121-131.

Harvey J.M., Uota M. (1978). Table grapes and refrigeration: fumigation with sulphur dioxide. International Journal of Refrigeration 1, 167-172.

Haselgrove L., Botting D., Van Heeswijck R. et al. (2000). Canopy, microclimate and berry composition: the effect of bunch exposure on the phenolics composition of *Vitis vinifera* L. cv. Shiraz grape berries. Australian Journal of Grape Wines Research 6: 141-149.

Hedin P.A., Jenkins J.N., Collum D.H., et al. (1983). Cyanidin-3-beta-glucoside, a newly recognized basis for resistance in cotton to the tobacco budworm *Heliothis virescens* (Fab) (Lepidoptera, Noctuidae). Experientia 39 (7): 799-801.

Hertog M.G.L., Hollman P.C.H., Katan M.B. (1992). Content of potentially anticarcenogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. Journal of Agricultural and Food Chemistry 40: 2379-2383.

Hincha D.K., Meins F.Jr., Schmitt J.M. (1997). β-1,3-glucanase is cryoprotective in vitro and is accumulated in leaves during cold acclimation. Plant Physiology 114: 1077-1083.

Hinton D.M., Pressey R. (1980). Glucanases in fruits and vegetables. Journal of the American Society for Horticultural Science 105 (4): 499-502.

Hird D.L., Worrall D.L., Hodge R., Smartt S., Paul W., Scott R. (1993). The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* A6 gene displays similarity to β-1,3-glucanases. Plant Journal 4: 1023-1033.

Hofacher W., Alleweldt G., Khader S. (1976). Einfluss von umweltfaktoren aut Beenrenwachstum und must qualitat bei der rebe. Vitis 15: 96-112.

Hoj P.B., Fincher G.B. (1995). Molecular evolution of plant beta-glucan endohydrolases. Plant Journal 7 (3): 367-379.

Holcroft D.M., Gil M.I., Kader A.A. (1998). Effect of carbon dioxide on anthocyanins, phenylalanine ammonia lyase and glucosyltransferase in the arils of stored pomegranates. Journal of the American Society for Horticultural Sciences 123: 136-140.

Holton T.A., Cornish E.C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7: 1071-83.

Hon W.C., Griffith M., Chong P.L., et al. (1994). Extraction and isolation of antifreeze proteins from winter rye (*Secale cereale*) leaves. Plant Physiology 104 (3): 971-980.

Hon W.C., Griffith M., Chong P.L., et al. (1994). Extraction and isolation of antifreeze proteins from winter rye (*Secale cereale* L.) leaves. Plant Physiology 104 (3): 971-980.

Hon W.C., Griffith M., Mlynarz A., et al. (1995). Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiology 109 (3): 879-889.

Hoos G., Blaich R.J. (1990). Influence of resveratrol on germination of conidia and mycelial growth of *Botrytis cinerea* and *Phomopsis viticola*. Journal Phytopathology 129: 102-110.

Houde M., Daniel C., Lachapelle M., Allard F., LaLiberte J-F, Sarhan F. (1995). Immunolocalization of freezing tolerance-associated proteins in the cytoplasm and nucleoplasm of wheat crown tissues. Plant Journal 8:583-593.

Howard L.R., Pandjaitan N., Morelock T., et al. (2002). Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. Journal of Agricultural and Food Chemistry 50 (21): 5891-5896.

Hrmova M., Fincher G.B. (1993). Purification and properties of 3 (1-]3)-beta-d-glucanase isoenzymes from young leaves of barley (*Hordeum vulgare*). Biochemical Journal 289: 453-461.

Hughes M.A., Dunn M.A., Pearce R.S., White A.J., Zhang L. (1992). An abcisic-acid-responsive low temperature barley gene has homology with a maize phospholipids transfer protein. Plant Cell and Environment 15: 861-865.

Hung L. M., Chen J. K., Huang S. S., Lee R. S., Su M. J. (2000). Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovascular Research 47: 549-555.

Hyodo H., Tanaka K., Suzuki T. (1991). Wound-induced ethylene synthesis and its involvement in enzyme induction in mesocarp tissue of *Cucurbita maxima*. Postharvest Biology and Technology 1: 127–136.

Ishikawa A., Tsubouchi H., Iwasaki Y., Asahi T. (1995). Molecular cloning and characterization of a cDNA for the α subunit of a G- protein from rice. Plant Cell Physiology 36: 353–359.

Jach G., Bornhardt B., Mundy J., Logemann J., Pinsdorf E., Leah R., Schell J., Maas C. (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant Journal 8: 97-109.

Jackman R.L., Smith J.L. (1996). Anthocyanins and Betalains. In: Natural Food Colorants. Hendry G.A.F. and Houghton J.D., eds. Blackie and Son, Ltd. London. pp. 244-309.

Jackman R.L., Yada R.Y., Marangoni A., et al. (1989). Chilling Injury - a review of quality aspects. Journal of Food Quality 11 (4): 253-278.

Jacobs A.K., Dry I.B., Robinson S.P. (1999). Induction of different pathogenesis-related cDNAs in grapevine infected with powdery mildew and treated with ethephon. Plant Pathology 48: 325-336.

Jacobs M. and Rubery P.H. (1988). Naturally-Ocurring Auxin Transport Regulators. Science 241: 346-349.

Jaglo-Ottosen K.R., Gilmour S.J., Zarka D.G., Schabenberger O., Thomashow M.F. (1998). *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science 280: 104-106.

Jakobek L., Seruga M., Medvidovic-Kosanovic M., et al. (2007). Anthocyanin content and antioxidant activity of various red fruit juices. Deutsche Lebensmittel-Rundschau 103 (2): 58-64.

Janas K.M., Cvikrova M., Palagiewicz A., et al. (2000). Alterations in phenylpropanoid content in soybean roots during low temperature acclimation. Plant Physiology and Biochemistry 38 (7-8): 587-593.

Janas K.M., Cvikrova M., Palagiewicz A., Szafranska K. and Posmyk M.M. (2002). Constitutive elevated accumulation of phenylpropanoids in soybean roots al low temperature. Plant Science 163: 369-373.

Janda T., Szalai G., Tari I., et al. (1999). Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. Planta 208 (2): 175-180.

Jang M., Cai L., Udeani G.O., Slowing K. V., Thomas C. F., Beecher C. W., Fong H. H., Farnsworth N. R., Kinghorn A. D., Mehta R. G., Moon R. C., Pezzuto J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275: 218-220.

Jeandet P., Bessis R., Sbaghi M., Meunier P. (1995). Production of the phytoalexin resveratrol by grape as a response to *Botrytis cinerea* attacks under natural conditions. Journal of Phytopathology 143: 135-139.

Ji C., Norton R.A., Wicklow D.T., et al. (2000). Isoform patterns of chitinase and beta-1,3-glucanase in maturing corn kernels (*Zea mays* L.) associated with *Aspergillus flavus* milk stage infection. Journal of Agricultural and Food Chemistry 48 (2): 507-511.

Jiang Y.M., Joyce D.C., Terry L.A. (2001). 1-Methylcyclopropene treatment affects strawberry fruit decay. Postharvest Biology and Technology 23 (3): 227-232.

Jiang Y.M., Zhang Z.Q., Joyce D.C., et al. (2002). Postharvest biology and handling of longan fruit (*Dimocarpus longan* Lour.) Postharvest Biology and Technology 26 (3): 241-252.

Jongedijk E., Tigelaar H., van Roekel J.S.C., Bres-Vloemans S.A., Dekker I., van den Kassanis B., Gianinazzi S., White R.F. (1995). A possible explanation of the resistance of virus-infected tobacco to second infection. Journal of Genetics and Virology 23: 11-16.

Jordao A.M., Ricardo-da-Silva J.M., Laureano O. (2001). Evolution of proanthocyanidins in bunch stems during berry development (*Vitis vinifera* L.) Vitis 40 (1) 17-22.

Juszczuk I.M., Wiktorowska A., Malusa E. and Rychter A.M. (2004). Changes in the concentration of phenolic compounds and exudation induced by phosphate deficiency in bean plants (*Phaseolus vulgaris* L.) Plant and Soil 267: 41-49.

Kacperska A. (1989). Metabolic consequences of low temperature stress in chilling-insensitive plants, In: Paul H.L. (Ed.), Low Temperature Stress Physiology in Crops, CRC Press Inc., Boca raton, Florida, pp. 27-40.

Kader A.A (2002). Postharvest biology and technology: an overview: In: Kader A.A. (Ed.), Postharvest Technology of Horticultural Crops, vol. 3311, third ed. University of California Agriculture Pub., USA, pp. 39-47.

Kallithraka S., Mohdaly A.A.A., Makris D.P., et al. (2005). Determination of major anthocyanin pigments in hellenic native grape varieties (*Vitis vinifera* sp.): Association with antiradical activity. Journal of Food Composition and Analysis 18 (5): 375-386.

Kalt W. (2005). Effects of production and processing factors on major fruit and vegetable antioxidants. Journal of Food Science 70: 11-19.

Kalt W., Forney C.F., Martin A. et al. (1999). Antioxidant capacity, vitamin C, phenolic, and anthocyanins after fresh storage of small fruits. Journal of Agricultural and Food Chemistry 47: 4638-4644.

Kalt W., McDonald J.E. (1996). Chemical composition of lowbush blueberry cultivars. Journal of the American Society for Horticultural Science 121: 142-146.

Karabulut O.A., Romanazzi G., Smilanick J.L., Lichter A. (2005). Postharvest ethanol and potassium sorbate treatments of table grapes to control gray mold. Postharvest Biology and Technology 37: 129-134.

Karabulut O.A., Smilanick J.L., Gabler F.M., Mansour M., Droby S. (2003). Near-harvest applications of Metschnikowia fructicola, ethanol, and sodium bicarbonate to control postharvest diseases of grape in central California. Plant Disease 87: 1384-1389.

Kassanis B., Gianinaz S., White R.F. (1974). Possible explanation of resistance of virus-infected tobacco plants to second infection journal of general virology 23: 11-16.

Kasukabe Y., He L.X., Nada K., Misawa S., Ihara I. and Tachibana S. (2004). Overexpression of spermidine synthase enhances tolerante to multiple environmental stresses and up-regulates the expression of various stress regulated genes in transgenic *Arabidopsis thaliana*. Plant Cell Physiology 45: 712-722.

Kazuoka T., Oeda K. (1994). Purification and characterization of cor85-oligomeric complex from cold-acclimated spinach. Plant Cell Physiology 35: 601-611.

Ke D., Saltveit M.E. (1989). Wound-induced ethylene production, phenolic metabolism and susceptibility to russet spotting in iceberg lettuce. Physiologia Plantarum 76 (3): 412-418.

Kim I.J., Chung W.I. (1998). Molecular characterization of a cytosolic ascorbate peroxidase in strawberry fruit. Plant Science 133 (1): 69-77.

Kim J.Y., Lee S.C., Jung K.H., et al. (2004). Characterization of a cold-responsive gene, OsPTR1, isolated from the screening of beta-glucuronidase (GUS) gene-trapped rice. Journal of Plant Biology 47 (2): 133-141.

Kim Y.J., Hwang B.K. (1994). Differential accumulation of β-1,3 glucanase and chitinase isoforms in pepper stems infected by compatible and incompatible isolates of Phytophthora capsici. Physiological and Molecular Plant Pathology 45: 95.

Kirubakaran S.I., Sakthivel N. (2007). Cloning and overexpression of antifungal barley chitinase gene in *Escherichia coli*. Protein Expression and Purification 52 (1): 159-166.

Kliewer W.M. (1970). Effect of day temperature and light intensity on coloration of *Vitis vinifera* L. grapes. Journal of the American Society for Horticultural Science 95:693–697.

Kodama H., Horiguchi G., Nishiuchi T., Nishimura M., Iba K. (1995). Fatty-acid desaturation during chilling acclimation is one of the factors involved in conferring low-temperature tolerance to young tobacco-leaves. Plant Physiology 107: 1177-1185.

Kombrink E., Somssich I.E. (1997). Pathogenesis-related proteins and plant defense. In: Carrol G.C., Tudzynsky P.U., eds. The mycota V, part A. Berlin:Springer-Verlag, pp. 107-127.

Lafuente M.T., Martínez-Tellez M.A., Zacarias L. (1997). Abcisic acid in the response of "Fortune" mandarin to chilling. Effect of madurity and high temperature conditioning. Journal of the Science of Food and Agricultural 73:494-502.

Lafuente M.T., Sala J.M. (2002). Abscisic acid and the influences of thylene, humidity and temperature on the incidence of postharvest rindstaining of navelina oranges (*Citrus sinensis* L. Osbeck) fruits. Postharvest Biology and Technology 25: 49-57.

Lafuente M.T., Zacarias L., Martínez-Téllez M.A., Sanchez-Ballesta M.T., Dupille E. (2001). Phenylalanine ammonia-lyase as related to ethylene in the development of chilling symptoms during cold storage of citrus fruits. Journal Agricultural and Food Chemistry 49: 6020-6025.

Lagunas-Solar M., Demateo A., Fernández J.E., Ozyarzum J.I., Carvacho O.F., Arancibia R.A., Delgado P.O. (1992). Radiotracer studies on the uptake and retention (conversion) of ^{35}S-sulfur dioxide in table grapes. American Journal of Enology and Viticulture 43(3): 266–274.

Lam S.K., Ng T.B. (2001). Isolation of a small chitinase-like antifungal protein from Panax notoginseng (*Sanchi ginseng*) roots. International Journal of Biochemistry and Cell Biology 33(3): 287-292.

Lamb C. J., Lawton M. A., Dron M., Dixon R. A. (1989). Signals and transduction mechanisms for activation of plant defences against microbial attack. Cell 56: 215-224.

Langcake P. (1981). Disease resistance of *Vitis* spp. And the production of the stress metabolites resveratrol, ε-viniferin, α-viniferin and pterostilbene. Physiology and Plant Patholology 18: 213-226.

Langcake P., McCarthy W.V. (1979).The relationship of resveratrol production to infection of grapevine leaves by *Botrytis cinerea*. Vitis 18, 244-253.

Langcake P., Pryce R.J. (1976). Production of resveratrol by *Vitis vinifera* and other members of *Vitaceae* as a response to infection or injury. Physiological Plant Pathology 9 (1): 77-86.

Langcake P., Pryce R.J. (1977). A new class of phytoalexins from grapevines. Experientia 33: 151-152.

Larson E.L., Morton H.E. (1991). Alcohols. In: S.S. Block, Editor, Disinfection, Sterilization, and Preservation (4th ed.), Lea and Febiger, London, pp. 191–203.

Lavee S., Nir G. (1986). Grape, In CRC Handbook of fruit set and development, (ed S.P. Monselise), CRC Press, Boca Raton, F.L., pp. 167-191.

Lawrence C.B., Singh N.P., Qiu J.S., et al. (2000). Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria solan*i and may function as an elicitor release mechanism. Physiological and Molecular Plant Pathology 57 (5): 211-220.

Lawton K., Ward E., Payne G., et al. (1992). Acidic and basic class-III chitinase messenger-RNA accumulation in response to tmv infection of tobacco. Plant Molecular Biology 19 (5): 735-743.

Lee B., Henderson D.A., Zhu J.K. (2005). The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. The Plant Cell 17: 3155-3175.

Lee S.W., Heinz R., Robb J., Nazar R.N. (1994). Differential utilization of alternate initiation sites in a plant defense gene responding to environmental stimuli. European Journal of Biochemistry 226: 109-114.

Lee Y.K., Hippe-Sanwald S., Jung H.W., Hong J.K., Hause B., Hwang, B.K. (2000) *In situ* localization of chitinase mRNA and protein in compatible and incompatible interactions of pepper stems with *Phytophthora capsici*. Physiology and Molecular Plant Pathology 57: 111-121.

Lei D.F., Feng Y., Jiang D.Z. (2004). Characterization of polyphenol oxidase from plants. Progress in Natural Science 14: 553-561.

Lewis N.G., Yamamoto E. (1990). Lignin- occurrence, biogenesis and biodegradation. Annual Review of Plant Physiology and Plant Molecular Biology 41: 455-496.

Leyva A., Jarillo J.A., Salinas J., Martinez-Zapater J.M. (1995). Low-temperature induces the accumulation of *phenylalanine ammonia-lyase* and *chalcone synthase* mRNAs of *Arabidopsis thaliana* in a light-dependent manner. Plant Physiology 108: 39-46.

Li J., Ou-Lee T., Raba R., Amundson R.G., Last R.(1993). *Arabidopsis* flavonoid mutant are hypersensitive to UV-B irradiation. Plant Cell 5: 171-179.

Liakopoulos G., Karabourniotis G. (2005). Boron deficiency and concentrations and composition of phenolic compounds in *Olea europaea* leaves: a combined growth chamber and field study. Tree Physiology 25: 307-315.

Lietti A., Cristoni A., Picci M. (1976). Studies on *Vaccinium myrtillus* anthocyanosides.1. Vasoprotective and anti-inflammatory activity. Arzneimittel-Forschung/Drug Research 26 (5): 829-832.

Lilie H., Schwarz E., Rudolph R.(1998). Advances in refolding of proteins produced in *E. coli*. Current Opinion in Biotechnology 9: 497-501.

Linderstrom-Lang K.U., Schellman J.A. (1959). Protein structure and enzyme activity. In: The enzymes, vol 1. Boyer P.D., Lardy H. and Myrback K. (eds.). Academic Press, New York, pp 443.

Linthorst H.J.M. (1991). Pathogenesis-related proteins of plants. Critical Reviews in Plant Sciences 10 (2): 123-150.

Lipton W.J., Wang C.Y. (1987). Chilling exposure and ethylene treatment change the levels of ACC in 'Honey Dew' melons. Journal of American Society Horticultural Science 112:109-112.

Liswidowati, Melchior F. Hohmann F., Schwer B., Kindl H. (1991). Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. Planta 183: 307-314.

Little C.R., Magill C.W. (2003). Elicitation of defense response genes in sorghum floral tissues infected by *Fusarium thapsinum* and *Curvularia lunata* at anthesis. Physiological and Molecular plant pathology 63 (5): 271-279.

Liu W.T., Chu C.L., Zhou T. (2002). Thymol and acetic acid vapors reduce post harvest brown rot of apricot and plums. HortScience 37: 151-156.

Lo Piero A.R., Puglisi I., Rapisarda P., Petrone G. (2005). Anthocyanins accumulation and related gene expression in red orange fruit induced by low temperature storage. Journal of Agricultural and Food Chemistry 53 (23): 9083-9088.

Loake G.J., Choudhary A.D., Harrison M.J., Mavandad M., Lamb C.J., Dixon R.A., (1991). Phenylpropanoid pathway intermediates regulate transient expression of a *chalcone synthase* gene promoter. Plant Cell 3: 829-840.

Lois R. (1994). Accumulation of UV-absorbing flavonoids induced by UV-B radiation in *Arabidopsis thaliana* L. I. Mechanisms of UV-resistance in Arabidopsis. Planta 194: 498-503.

Lois R., Dietrich A. and Hahlbrock K. (1989). A phenylalanine ammonia-lyase gene from parsley:structure, regulation and identification of elicitor and light responsive *cis*-acting elements. European Molecular Biology Organization Journal 8: 1641-1648.

Lu B.B., Du Z., Ding R.X., et al. (2006). Cloning and characterization of a differentially expressed phenylalanine ammonialyase gene (liPAL) after genome duplication from tetraploid I*satis indigotica* Fort. Journal of Integrative Plant Biology 48 (12): 1439-1449.

Lurie S., Klein J.D. (1991). Acquisition of low-temperature tolerance in tomatoes by exposure to high-temperature stress. Journal of the American Society for Horticultural Science 116 (6): 1007-1012.

Lurie S., Ronen R., Lipsker Z. and Aloni B. (1994). Effects of paclobutrazol and chilling temperatures on lipids, antioxidants and ATPase activity of plasma membrane isolated from green bell pepper fruits. Physiologia Plantarum 91:593-598.

Mahajan P.V., Goswami T.K. (2007). Use of liquid nitrogen in CA storage: theoretical analysis and experimental validation. Journal of Food Engineering, 82: 77-83.

Maher E.A., Bate N.J., Ni W., Elkind Y., Dixon R.A., Lamb C.J. (1994). Increased disease susceptibility of transgenic tobacco plants with suppressed levels pf preformed phenylpropanoid products. Proceedings of the National Academy of Science in U.S.A. 91: 7802-7806.

Maldonado R., Goni O., Escribano M.I., et al. (2007). Regulation of phenylalanine ammonia-lyase enzyme in Annona fruit: Kinetic characteristics and inhibitory effect of ammonia. Journal of Food Chemistry 31 (2): 161-178.

Maldonado R., Molina-Garcia A.D., Sanchez-Ballesta M.T., et al. (2002). High CO2 atmosphere modulating the phenolic response associated with cell adhesion and hardening of Annona cherimola fruit stored at chilling temperature. Journal of Agricultural and Food Chemistry 50 (26): 7564-7569.

Mansfield J.W., Hutson R.A. (1980). Microscopical studies on fungal development and host responses in broad bean and tulip leaves inoculated with 5 species of *Botrytis*. Physiological Plant Pathology 17 (2): 131.

Manteau S., Abouna S., Lambert B., et al. (2003). Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. FEMS Microbiology Ecology 43 (3): 359-366.

Martínez-Romero D., Guillén F., Castillo S., Valero D., Serrano M. (2003). Modified atmosphere packaging maintains quality of table grapes. Journal of Food Science 68: 1838-1843.

Martinez-Romero D., Valero D., Serrano M., et al. (2000). Exogenous polyamines and gibberellic acid effects on peach (*Prunus persica* L.) storability improvement. Journal of Food Science 65 (2): 288-294.

Martinez-Tellez M.A., Lafuente M.T. (1997). Effect of high temperature conditioning on ethylene, phenylalanine ammonia-lyase, peroxidase and polyphenol oxidase activities in flavedo of chilled 'Fortune' mandarin fruit. Journal of Plant Physiology 150 (6): 674-678.

Masia A. (1998). Superoxide dismutase and catalase activities in apple fruit during ripening and post-harvest and with special reference to ethylene. Physiologia Plantarum 104 (4): 688-672.

Mauch F., Staehelin L.A. (1989). Functional implications of the subcellular-localization of ethylene-induced chitinase and beta-1,3-glucanase in bean-leaves. Plant Cell 1 (4): 447-457.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiology 88: 936-942.

Mazza, G., Maniati, E. (1993). Anthocyanins in Fruits, Vegetables and Grains. Boca Raton, FL: CRC Press, pp. 85-87.

Meins F., Ahl P. (1989). Induction of chitinase and beta-1,3-glucanase in tobacco plants infected with *Pseudomonas-tabaci* and *Phytophthora-parasitica* var. Nicotianae. Plant Science 61 (2): 155-161.

Meins F., Neuhaus J.M., Sperisen C., Ryals J. (1992). The primary structure of plant pathogenesis-related glucanohydrolases and their genes, in: T. Boller, F.Jr. Meins, (Eds.), Genes Involved in Plant Defense, Vienna, Springer-Verlag, New York, pp. 245-282.

Meins M., Jeno P., Muller D., et al. (1993). Cysteine phosphorylation of the glucose transporter of *Escherichia coli*. Journal of Biological Chemistry 268(16): 11604-11609.

Meir S., Philosoph-Hadas S., Lurie S., Droby S., Akerman M., Zuberman G., Shapiro B., Cohen E. and Fuchs Y. (1996). Reduction of chilling injury in stored avocado, grapefruit, and bell pepper by methyl jasmonate. Canadian Journal of Botany 74:870-874.

Melchers L.S., Apothekerdegroot M., Vanderknaap J., et al. (1994). A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. Plant Journal 5 (4): 469-480.

Melchior F., Kindl H. (1990). Grapevine stilbene synthase cdna only slightly differing from chalcone synthase cdna is expressed in escherichia-coli into a catalytically active enzyme. FEBS Letters 268 (1): 17-20.

Merodio C., De La Plaza J.L. (1997). Cherimoya. In: Mitra S.K., ed. Postharvest physiology and storage of tropical and subtropical fruits. Wallingford: CAB International, pp. 265-290.

Merodio C., Muñoz M.T., Del Cura B., Buitrago D., Escribano M.I. (1998). Effect of high CO2 level of the tigres of γ-aminobutyric acid, total polyamines and some patogénesis-related proteins in chirimoya fruit stored at low temperature. Journal of Experimental Botany 49 (325): 1339-1347.

Metraux J.P., Burkhart W., Moyer M., et al. (1989). Isolation of a complementary-DNA encoding a chitinase with structural homology to a bifunctional lysozyme chitinase. Proceedings of the National Academy of Sciences of the United States of America 86 (3): 896-900.

Miceli A., Ippolito A., Linsalata V., Nigro F., (1999). Effect of preharvest calcium treatments on decay and biochemical changes in table grape during storage. Phytopathologia Mediterranea 38: 47–53.

Michalski W.P., Kaniuga Z. (1981). Photosynthetic apparatus of chilling-sensitive plants. X. Relationship between superoxide dismutase activity and photoperoxidation of chloroplast lipid. Biochimica et Biophysica Acta 637:159.

Middelberg A.P.J. (2004). Preparative protein folding. Trends in Biotechnology 20: 433-437.

Miller N.J., Sampson J., Candeias L.P., Bramley P.M., Rice-Evans C.A. (1996). Antioxidant activities of carotenes and xanthophylls. FEBS Letters 384: 240-242.

Mittler R., Zilinskas B.A. (1991). Purification and characterization of pea cytosolic ascorbate peroxidase. Plant Physiology 97 (3): 962-968.

Mol J.N.M., Stuitje A.R., Vanderkrol A. (1989). Genetic manipulation of floral pigmentation genes. Plant Molecular Biology 13(3): 287-294.

Moline H.E., Buta J.G., Saftner R.A., Maas J.L. (1997). Comparison of three volatile natural products for the reduction of post harvest diseases in strawberries. Advances in Strawberry Research 16: 43-48.

Monroy A.F., Dhindsa R.S. (1995). Low temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25ºC. Plant Cell 7: 321-331.

Montero C., Cristescu S.M., Jiménez J.B., Orea J.M., te Lintel Hekkert S., Harren F.J.M. and González Ureña A. (2003). *Trans*-Resveratrol and Grape Disease Resistance. A Dynamical Study by High-Resolution Laser-Based Techniques. Plant Physiology 131: 129-138.

Morohashi Y., Matsushima H. (2000). Development of beta-1,3-glucanase activity in germinated tomato seeds. Journal of Experimental Botany 51 (349): 1381-1387.

Morris J.R. (1982). Effects of preharvest practices on the quality of grape juice. Abstracts of Papers of the American Chemical Society 184: 40

Morris J.R., Oswald O.L., Main G.L., Moore J.N., Lark J.R. (1992). Storage of new seedless grape cultivar with sulphur dioxide generators. American Journal of Enology and Viticulture 43 (3): 230-232.

Moyls A.L., Sholberg P.L. and gaunce A.P. (1996). Modified-atmosphere packaging of grapes and strawberries fumigated with acetic acid. Hortscience 31 (3): 414-416.

Muñoz M.T., Aguado P., Ortega N., escribano M.I., Merodio C. (1999). Regulation of ethylene and polyamine síntesis by elevated carbon dioxide in chirimoya fruit stored at ripening and chilling temperatures. Australian Journal of Plant Physiology 26: 201-209.

Musetti R., Marabottini R., Badiani M., Martini M., di Toppi L.S., Borselli S., Borgo M., Osler R. (2007). On the role of H2O2 in the recovery of grapevine (*Vitis vinifera* cv. Prosecco) from Flavescence doree disease. Functional Plant Biology 34 (8): 750-758.

Nagy Z., Tuba Z., Zsoldos F., et al.(1995). CO2-exchange and water relation responses of sorghum and maize during water and salt stress. Journal of Plant Physiology 145 (4): 539-544.

NDong C, Anzellotti D, Ibrahim RK, et al. (2003). Daphnetin methylation by a novel O-methyltransferase is associated with cold acclimation and photosystem II excitation pressure in rye. Journal of Biological Chemistry 278 (9): 6854-6861.

Nelson K.E. (1979). Harvesting and handling California table grapes for market. University of California, Publication 4095.

Nelson K.E., Ahmedullah M. (1972). Effect of type of in-package sulphur dioxide generator and packaging materials on quality of stored table grapes. American Journal of Enology and Viticulture 23: 78-85.

Nelson K.E., Ahmedullah M. (1976). Effect of the temperature change on the release rate of sulphur dioxide from two-stage sodium bisulfite generators. American Journal of Enology and Viticulture 24: 75-80.

Neuhaus J.M., Shinshi H., VanBuuren M., et al. (1987). Characterization of tobacco chitinase genes. Experientia 43 (6):662-662.

Neuhaus J.M., Sticher L., Meins F.Jr., Boller T. (1991). A short C-terminal sequence is necessary and sufficient for the targeting of chitinase to the plant vacuole. Proceedings of the National Academy of Science in U.S.A 88: 10362-10366.

Neuhaus, J.M. (1999) Plant chitinases (PR-3, PR-4, PR-8, PR-11). In "Pathogenesisrelated proteins in plants", eds. Datta, S.K. and Mathukrishnan, S., CRC Press, Boca Raton, pp. 77-105.

Newton S.S., Duman, J.G. (2000). An osmotin-like cryoprotective protein from the bittersweet nightshade *Solanum dulcamara*. Plant Molecular Biology 44: 581-589.

Nguyen T.B.T., Ketsa S., van Doorn W.G. (2003). Relationship between browning and the activities of polyphenol oxidase and phenylalanine ammonia lyase in banana peel during low temperature storage. Postharvest Biology and technology 30: 187-193.

Nurnberger T., Scheel, D. (2001). Signal transmission in the plant immune response. Trends in Plant Science 6: 372-379.

Nursten H.E. (1970). Volatile compounds. The aroma of fruits. In: Hulme A.C. (Ed.), The biochemistry of Fruits and Their Products. Academic Press, new York, pp. 239-268.

O´Riordain G., Radauer C., Hoffmann-Sommergruber K., Adhami, F., Peterbauert C.K., Blanco C., Godnie-Cvar J., Scheiner O., Ebner C. and Breiteneder H. (2002). Cloning and molecular characterization of the *Hevea brasiliensis* allergen Hev b 11, a class I chitinase. Clinical Experimental Allergy 32:455-462.

Oak M.H., Bedoui J.E., Madeira S.V. et al. (2006). Delphinidin and cyanidin inhibit PDGF(AB)-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK. British Journal of Pharmacology 149:283–290.

Ohl S., Hedrick S.A, Chory J., Lamb C.J. (1990). Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2: 837-848.

Orak H.H. (2007). Total antioxidant activities, phenolics, anthocyanins, polyphenoloxidase activities of selected red grape cultivars and their correlations. Scientia Horticulturae 111: 235-241.

Oren-Shamir M., Levi-Nissim A. (1997). UV-light effect on the leaf pigmentation of *Cotinus coggygria* 'Royal Purple'. Scientia Horticulturae 71 (1-2): 59-66.

Ori N., Sessa G., Lotan T., Himmelboch S., Fluhr R. (1990). A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass. EMBO Journal 9: 3249-3436.

Ose K., Chachin K., Imahori Y. (1995). Browning Mechanism of Water Convolvulus (Ipomoea-Aquativa Forsk) Stored at Low-Temperature. Enzymatic Browning and its Prevention 600: 178-187.

Ozgen M., Reese R.N., Tulio Jr. A.Z., Scheerens J., Miller A.R. (2006). Modified 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Method to Measure Antioxidant Capacity of Selected Small Fruits and Comparison to Ferric Reducing Antioxidant Power (FRAP) and 2,2´-Diphenyl-1-picrylhydrazyl (DPPH) Methods. Journal of Agricultural and Food Chemistry 54: 1151-1157.

Pastirova A., Repcak M., Eliasova A. (2004). Salicylic acid induces changes of coumarin metabolites in *Matricaria chamomilla* L. Plant Science 167 (4): 819-824.

Patterson B.D., Kenrick J.R., Raison J.K. (1978). Lipids of chill-sensitive and chill-resistant passiflora species - fatty-acid composition and temperature-dependence of spin label motion. Phytochemistry 17 (7): 1089-1092.

Perez-Jimenez J., Saura-Calixto F. (2006). Effect of solvent and certain food constituents on different antioxidant capacity assays. Food Research International 39 (7): 791-800.

Pieterse C.M.J., van Loon L.C. (1999). Salicylic acid-independent plant defence pathways. Trends in Plant Science 4 (2): 52-58.

Poley G.E., Slater J.E. (2000). Latex allergy. Journal of Allergy Clinical Immunology 105: 1054-62.

Porat R., Vinokur V., Holland D., McCollum T.G., Droby S. (2001). Isolation of a citrus chitinase cDNA and characterization of its expression in response to elicitation of fruit pathogen resistance. Journal of Plant Physiology 158: 1585-1590.

Porat R., Weiss B., Cohen L., Daus A., Goren R. and Droby S. (1999). Effects of ethylene and 1 methylcyclopropene on the postharvest qualities of 'Shamouti' oranges. Postharvest Biology and Technology 15: 155-163.

Pretel M.T., Martínez-Madrid M.C., Martínez J.R., Carreño J.C., Romojaro F. (2006). Prolonged storage of 'Aledo' table grapes in a slightly CO2 enriched atmosphere in combination with generators of SO2. Lebensmittel-Wissenschaft und Technologie 39: 1109-1116.

Price M.V., Waser N.M. (1998). Effects of experimental warming on plant reproductive phenology in a subalpine meadow. Ecology 79 (4): 1261-1271.

Priestley D.A., Leopold A.C. (1979). Relevance of seed membrane-lipids to imbibitional chilling damage. Plant Physiology 63 (5): 68-68.

Prusky D. (1996). Pathogen quiescence in postharvest diseases. Annual Review of Phytopathology 34: 413-434.

Prusky D., Lichter A. (2007). Activation of quiescent infections by postharvest pathogens during transition from the biotrophic to the necrotrophic stage. FEMS Microbioly Letter 268: 1-8.

Punja Z.K., Zhang Y.Y. (1993). Plant chitinases and their roles in resistance to fungal disease. Journal of Nematology 25: 526–540.

Raison J.K., Lyons J.M. (1986). Chilling injury - a plea for uniform terminology. Plant Cell and Environment 9 (9): 685-686.

Raison J.K., Orr G.R. (1986). Phase-transitions in thylakoid polar lipids of chilling-sensitive plants - a comparison of detection methods. Plant Physiology 80 (3): 638-645.

Raison J.K., Wright L.C. (1983). Thermal phase-transitions in the polar lipids of plant membranes - their induction by disaturated phospholipids and their possible relation to chilling injury. Biochimica et Biophysica Acta 731 (1): 69-78.

Ray P.K., Ruby R., Singh S.K. (2005). Effect of sulphur dioxide fumigation and low temperature storage on post-harvest browning and quality of litchi fruits. Journal of Food Science and Technology_Mysore 42 (3): 226-230.

Receveur-Bréchot V., Czjzek M., Barre A., Roussel A., Peumans W.J., Van Damme E.J.M., Rougé P. (2006). Crystal structure at 1.45-A Resolution of the Major Allergen Endo-β-1,3-Glucanase of Banana as a Molecular Basis for the Latex-fruit Syndrome. Proteins: Structure, Function and Bioinformatics 63: 235-242.

Redolfi P., Cantisani A. (1984). Preliminary characterization of new soluble-proteins in *Phaseolus-vulgaris* cv. Saxa reacting hypersensitively to viral-infection. Physiological Plant Pathology 25 (1): 9-19.

Remón S., Ferrer A., López-Buesa P., Oria R. (2004). Atmosphere composition effects on Burlat cherry colour during cold storage. Journal of the Science of Food and Agriculture 84: 140–146.

Remon S., Ferrer A., Marquina P., et al.(2000). Use of modified atmospheres to prolong the postharvest life of Burlat cherries at two different degrees of ripeness. Journal of the Science of Food and Agriculture 80 (10): 1545-1552.

Renault A.S., Deloire A., Bierne J. (1996). Pathogenesis –related proteins in grapevines induced by salicylic acid and *Botrytis cinerea*. Vitis 35: 49-52.

Retamales J., Defilippi B.G., Arias M., Castillo P., Manríquez D. (2003). High-CO_2 controlled atmospheres reduce decay incidence in Thompson Seedless and Red Globe table grapes. Postharvest Biology and Technology 29: 177-182.

Rezzonico E., Flury N., Meins F., et al. (1998). Transcriptional down-regulation by abscisic acid of pathogenesis-related beta-1,3-glucanase genes in tobacco cell cultures. Plant Physiology 117 (2): 585-592.

Rifai L.A., Koussa T., Fassouane A., et al. (2004). Development of polyamine levels in flower buds, flowers and young berries of *Vitis vinifera* L. (cv. Cabernet Sauvignon) infected by eutypiosis fungus, Eutypa lata. Vitis 43 (3): 139-144.

Riov J., Monselis S.P., Kahan R.S. (1969). Ethylene-controlled induction of phenylalanine ammonia-lyase in citrus fruit peel. Plant Physiology 44(5): 631.

Ritenour M.A., Saltveit M.E. (1996). Identification of a phenylalanine ammonia-lyase inactivating factor in harvested head lettuce (*Lactuca sativa*). Physiologia Plantarum 97 (2): 327-331.

Robert N., Roche K., Lebeau Y., Breda C., Boulay M., Esnault R., Buffard D. (2002). Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. Plant Science 162: 389-400.

Roberts W.K., Selitrennikov C.P. (1988). Plant and bacterial chitinases differ in antifungal activity. Journal of Genetic and Microbiology 134: 169-176.

Robinson S.P., Jacobs A.K., Dry I.B. (1997). A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiology 114 (3): 771-778.

Rodríguez-López J.N., Espin J.C., Tudela J., Martínez V., Cerda A., García-Canovas F. (2000). Purification and kinetic characterization of peroxidase from tomato cultivated under different salinity conditions. Journal of Food Science 65: 15-19.

Rogers E. E., Glazebrook J., Ausubel F. N. (1996). Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*-pathogen interactions. Molecular Plant Microbe Interactions 9:748-757.

Rollins J.A., Dickman M.B. (2001). pH signaling in *Sclerotinia sclerotiorum*: Identification of a pacC/RIM1 Homolog. Applied and Environmental Microbiology 67 (1): 75-81.

Rosemann D., Heller W., Sandermann H. Jr. (1991). Biochemical plant responses to ozone. II. Induction of stilbenes biosynthesis in Scots Pine (*Pinus sylvestris*) seedlings. Plant Physiology 97: 1280-1286.

Rothan C., Duret S., Chevalier C., et al. (1997). Suppression of ripening-associated gene expression in tomato fruits subjected to a high CO2 concentration. Plant Physiology 114 (1): 255-263.

Roubelakisangelakis K.A., Kliewer W.M. (1986). Effects of exogenous factors on phenylalanine ammonia-lyase activity and accumulation of anthocyanins and total phenolics in grape berries. American Journal of Enology and Viticulture 37 (4): 275-280.

Ruffner (1982). Metabolism of tartaric and malic-acids in *Vitis*. A review. A. Vitis 21(3): 247-259.

Ryan D., Antolovich M., Prenzler P., et al. (2002). Biotransformations of phenolic compounds in *Olea europaea* L. Scientia Horticulturae 92 (2): 147-176.

Ryu S.B., Li P.H. (1994). Potato cold hardiness development and abcisic acid. I. Conjugated abscisic acid is not the source of the increase in free abscisic acid during potato (*Solanum Commersoni*) cold acclimatation. Physiologia Plantarum 90: 15-20.

Sakamoto A., Murata N. (2002). The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant Cell and Environment 25 (2): 163-171.

Salveit (Jr) M.E., Morris L.L. (1990). Overview on chilling injury of horticultural crops. In: "Chilling injury of horticultural crops" (C.Y. Wang, ed), CRC Press, Boca Raton, Florida, pp. 3-15.

Salzman R.A., Tikhonova I., Bordelon B.P., et al. (1998). Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. Plant Physiology 117 (2): 465-472.

Sanchez-Ballesta M.T., Gosalbes M.J., Rodrigo M.J., Granell A., Zacarias L., Lafuente M.T. (2006). Characterization of a β-1,3-glucanase from citrus fruit as related to chilling-induced injury and ethylene production. Postharvest Biology and Technology 40: 133-140.

Sanchez-Ballesta M.T., Lafuente M.T., Zacarias L., Granell A. (2000). Involvement of phenylalanine ammonia-lyase in the response of Fortune mandarin fruits to cold temperature. Physiologia Plantarum 108: 382-389.

Sanchez-Ballesta M.T., Rodrigo M.J., Lafuente M.T., Granell A., Zacarias L. (2004). Dehydrin from citrus, which confers in vitro dehydration and freezing protection activity, is constitutive and highly expressed in the flavedo of fruit but responsive to cold and water stress in leaves. Journal of Agricultural and Food Chemistry 52: 1950-1957.

Sanchez-Monge R., Balnco C., Díaz-Perales A. et al. (1999). Isolation and characterization of major banaba allergens: identification as fruit class I chitinases. Clinical Experimental Allergy 29:673-80.

Santos I.S., Da Cunha M., Machado O.L.T., et al. (2004). A chitinase from *Adenanthera pavonina* L. seeds: purification, characterization and immunolocalization. Plant Science 167 (6): 1203-1210.

Sarig P., Zutkhi Y., Monjauze A., Lisker N., Ben-Arie R. (1997). Phytoalexin elicitation in grape berries and their susceptibility to *Rhizopus stolonifer*. Physiology and Molecular Plant Pathology 50: 337-347.

Sbaghi M., Jeandet P., Faivre B., Bessis R., Fournioux J. C. (1995). Development of methods using phytoalexin (resveratrol) assessment as a selection criterion to screen grapevine *in vitro* cultures for resistance to grey mould (*Botrytis cinerea*). Euphytica 86: 41-47.

Schijlen E.G.W., de Vos C.H.R., van Tunen A.J., et al. (2004). Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65 (19): 2631-2648.

Schmelzer E., Jahnen W., Hahlbrock K. (1988). *Insitu* localization of light-induced chalcone synthase messenger-RNA, chalcone synthase, and flavonoid end products in epidermal-cells of parsley leaves. Proceedings of the National Academy of Sciences of the United States of America 85 (9): 2989-2993.

Schubert R., Fischer R., Hain R., et al. (1997). An ozone-responsive region of the grapevine resveratrol synthase promoter differs from the basal pathogen-responsive sequence. Plant Molecular Biology 34 (3): 417-426.

Schultze M., Staehelin C., Brunner F., Genetet I., Legrand M., Fritig B., Kondorosi E., Kondorosi A. (1998). Plant chitinase/lysozyme isoforms show distinct substrate specificity and cleavage site preference towards lipochitooligosaccharide Nod signals. Plant Journal 16: 571-580.

Seki M., Narusaka M., Ishida J., Nanjo T., Fujita M., Oono Y., Kamiya A., Nakajima M., Enju A., Sakurai T. et al. (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold, and high-salinity stresses using a full-length cDNA microarray. Plant Journal 31: 279-292.

Seki M., Umezawa T., Urano K. and Shinozaki K. (2007). Regulatory metabolic networks in drought stress responses. Current Opinion in Plant Biology 10: 296-302.

Sela-Buurlage M.B., Ponstein A.S., Bres-Vloemans S.A., Melchers L.S., van den Elzen P.J.M., Cornelissen B.J.C. (1993). Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiology 101: 857-863.

Serrano M., Martínez-Madrid M.C., Martínez G., Riquelme F., Pretil M.T., Romojaro F. (1996). Role of polyamines in chilling injury of fruti and vegetables. Food Science and Technology International 2: 195-199.

Sgarbi E., Fornasiero R.B., Lins A.P., Bonatti P.M. (2003). Phenol metabolism is differentially affected by ozone in two cell lines from grape (*Vitis vinifera* L.) leaf. Plant Science 165: 951-957.

Shinshi H., Usami S., Ohme-Takagi M. (1995). Identification of an ethylene responsive region in the promoter of a tobacco class I chitinase gene. Plant Molecular Biology 27: 923-932.

Shinshi H., Wenzler H., Neuhaus J.M., et al. (1988). Evidence for N-terminal and C-terminal processing of a plant defense-related enzyme - primary structure of tobacco prepro-beta-1,3-glucanase. Proceedings of the National Academy of Sciences of U.S.A. 85 (15): 5541-5545.

Sholberg P.L., Gaunce A.P. (1995). Fumigation of fruit with acetic acid to prevent post harvest decay. HostScience 30: 1271-1275.

Sholberg P.L., Reynolds A.G., Gaunce A.P. (1996). Fumigation of table grapes with acetic acid to prevent post harvest decay. Plant Disease 80: 1425-1428.

Showalter A.M., Bell J.N., Cramer C.L., et al. (1985). Accumulation of hydroxyproline-rich glycoprotein messenger-RNAs in response to fungal elicitor and infection. Proceedings of the National Academy of Sciences of the United States of America 82 (19): 6551-6555.

Shulaev V., Silverman P., Raskin I., (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. Nature 385 (6618): 718-721.

Simmons C.R. (1994). Physiology and molecular-biology of plant 1,3-beta-d-glucanases and 1,3 1,4-beta-D-glucanases. Critical Review in Plant Science 13: 325-387.

Smith, C. J. 1996. Accumulation of phytoalexins: defence mechanism and stimulus response system. New Phytology 132: 1-45.

Sofos J.N. (1989). Sorbate Food Preservatives, CRC Press, Boca Raton, FL, p. 237.

Solecka D., Boudet A.-M., Kacperska A. (1999). Phenylpropanoid and anthocyanin changes in low temperature treated winter oilseed rape leaves. Plant Physiology and Biochemistry 37: 491-496.

Song J., Leepipattanawit R., Deng W., Beaundry R.M. (1996). Hexenal vapour is a natural, metabolizable fungicide: inhibition of fungal activity and enhancement of aroma biosynthesis in apple slices. Journal of American Society of Horticultural Science 121: 937-942.

Sowka S., Hsich L.S., Krebitz M., et al. (1998). Identification and cloning of *Prs a 1*, a 32 kDa endochitinase and major allergen of avocado, and its expression in the yeast *Pichia pastoris*. Journal of Biology and Chemistry 273: 28091-28097.

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C. (1994). Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Molecular Biology 24: 743-755.

Stewart R.J., Sawyer B.J.B., Bucheli C.S., Robinson S.P. (2001). Polyphenol oxidase is induced by chilling and wounding in pineapple. Australian Journal of Plant Physiology 28: 181-191.

Sticher L., Hofsteenge J., Milani A., Neuhaus J.-M., Meins F. Jr. (1992) Vacuolar chitinases of tobacco: A new class of hydroxyproline-containing proteins. Science 257: 655-657.

Stiles E.A., Cech N.B., Dee S.M., Lacey E.P. (2007). Temperature-sensitive anthocyanin production in flowers of *Plantago lanceolata*. Physiologia Plantarum 129 (4): 756-765.

Stintzi A., Heitz T., Prasad V., Wiedemann-Merdinoglu S., Kauffmann S., Geoffroy P., Legrand M., Fritig B. (1993). Plant 'pathogenesis-related' proteins and their role in defence against pathogens. Biochimie 75: 687-706.

Stintzing F.C., Carle R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. Trends in Food Science and Technology 15: 19–38.

Stone B.A., Clarke A.E. (1992). Chemistry and biology of (1,3)-ß-glucans. Victoria, Australia: La Trobe University Press.

Strack D., Wray V. (1994). In: Harbourne, J.B. (Ed.), The Flavonoids, Advances in research since 1986. Chapman and Hall, London, p. 1.

Strauss C.R., Dimitriadis E., Wilson B., et al. (1986). Studies on the hydrolysis of 2 megastigma-3,6,9-triols rationalizing the origins of some volatile c-13 norisoprenoids of *Vitis vinifera* grapes. Journal of Agricultural and Food Chemistry 34 (1): 145-149.

Suarez V., Staehelin C., Arango R., Holtorf H., Fofsteenge J., Meins F. Jr. (2001). Substrate specificity and antifungal activity of recombinant tobacco class I chitinases. Plant Molecular Biology 45: 609-618.

Subroto T., Sufiati S., Beintema J.J. (1999). Papaya (*Carica papaya*) lysozyme is a member of the family 19 (basic, class II) chitinases. Journal of Molecular Evolution 49: 819-821.

Sung D.Y., Kaplan F., Lee K.J., Guy C.L. (2003). Acquired tolerance to temperature extremes. Trends in Plant Science 8: 179-187.

Surinder M.S., Amulya K.P. (2005). Solubilization and refolding of bacterial inclusion proteins. Journal of Bioscience and Bioengineering 99 (4): 303-310.

Sutton F., Ding X., Kenefick D.G. (1992). Group-3 LEA gene Hva1 regulation by cold-acclimation and deacclimation in 2 barley cultivars with varying freeze resistance. Plant Physiology 99: 338-340.

Svensson A.S., Johnsson F.I., Moller I.M., Rasmusson A.G. (2002). Cold stress decreases the capacity for respiratory NADH oxidation in potato leaves. Febs Letters 517: 79-82.

Tahlil N., Rada A., Baaziz M., Morel J.L., El Meray M., El Aatmani M. (1999). Quantitative and qualitative changes in peroxidase of *Cucurbita pepo* cultivars stressed with heavy metals. Biologia Plantarum 42: 75-80.

Takahashi T., Abe K., Chachin K. (1996). Studies on the physiological and chemical changes in shredded cabbage .3. Effect of air-exposure at low temperature on physiological activities and browning of shredded cabbage. Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi 43 (6): 663-667.

Takakura Y., Ito T., Saito H., Inoue T., Komari T., Kuwata S. (2000). Flower predominant expression of a gene encoding a novel class I chitinase in rice (*Oryza sativa* L.) Plant Molecular Biology 42: 883-897.

Takayanagi T., Okuda T., Mine Y., Yokotsuka K. (2004). Induction of resveratrol biosynthesis in skins of three grape cultivars by ultraviolet irradiation. Journal of the Japanese Society for Horticultural Science 73 (3): 193-199.

Takeda F., Saunders M.S., Saunders J.A. (1983). Physical and chemical-changes in muscadine grapes during post-harvest storage. American Journal of Enology and Viticulture 34 (3): 180-185.

Tamagnone L., Merida A., Stacey N., Paskitt K., Parr A., Chang C.F., Lynn D., Dow J.M., Roberts K., Martin C. (1998). Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. Plant Cell 10: 1801-18016.

Tamiya T., Okahashi N., Sakuma R., et al. (1985). Freeze denaturation of enzymes and its prevention with additives. Cryobiology 22 (5): 446-456.

Tasgin E., Atici O., Nalbantoglu B. (2003). Effects of salicylic acid and cold on freezing tolerance in winter wheat leaves. Plant Growth Regulation 41 (3): 231-236.

Teutonico R.A., Dudley M.W, Orr J.D., et al. (1991). Activity and accumulation of cell division-promoting phenolics in tobacco tissue-cultures. Plant physiology 97 (1): 288-297.

Teutonico R.A., Dudley M.W., Orr J.D., Lynn D.G., Binns A.N. (1991). Activity and accumulation of cell division-promoting phenolics in tobacco tissue-cultures. Plant Physiology 97: 288-297.

Thalmair M., Bauw G., Thiel S., Dohring T., Langebartels C., Sandermann H. Jr. (1996). Ozone and ultraviolet B effect on the defense-related protein β-1,3-glucanase and chitinase in tobacco. Journal of Plant Physiology 148: 222-228.

Thimmapuram J., Ko T.-S., Korban S.S. (2001). Characterization and expression of ß-1,3-glucanase genes in peach. Molecular genetics and Genomics 265: 469-479.

Thomashow M.F. (1999). Plant cold tolerance: freezing tolerance genes and regulatory mechanisms. Annual Review of Plant Physiology and Plant Molecular Biology 50: 571-599.

Thomma B.P.H.J., Eggermont K., Broekaert W.F., et al. (2000). Disease development of several fungi on arabidopsis can be reduced by treatment with methyl jasmonate. Plant Physiology and Biochemistry 38 (5): 421-427.

Thulke O., Conrath U. (1998). Salicylic acid has a dual role in the activation of defence-related genes in parsley. Plant Journal 14 (1): 35-42.

Tiburcio A.F., Kaur-Sawhney R., Galston A.W. (1990). Polyamine Metabolism. In: The Biochemistry of Plants, A Comprehensive Treatise, Vol. 16. PkK. Stumpf. E.E. Conn, (eds) Academic Press, New York, pp 283-325.

Timberlake C.F., Bridle P. (1982). Distribution of anthocyanins in food plants. In: Anthocyanins as Food Colors (edited by P. Markakis), New York, NY: Academic Press, pp. 126–157.

Tomás-Barberán F., Espin J.C. (2001). Phenolic acmpounds and related enzymes as determinants of quality in fruits and vegetables. Journal of Science and Food Agriculture 81: 853-876.

Treshow M. (1989). Plant stress from air pollution. Michael Treshow and Franklin K. (Eds) p. 200.

Tripathi P., Dubey N.K. (2002). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. Postharvest Biology and Technology 32 (3): 235-245.

Tronsmo A.M., Gregersen P., Hjeljord L., Sandal T., Bryngelsson T., Collinge D.B. (1993). Cold-induced disease resistance. In: Fritig B., Legrand M., eds, Mechanisms of Plant Defense Responses. Kluwer Academic, The Netherlands, pp. 369-385.

Tsumoto K., Ejima D., Kumagai I., Arawaka T. (2003). Practical considerations in refolding proteins from inclusion bodies. Protein Expression and Purification 28:1-8.

Ukaji N., Kuwabara C., Takezawa D., et al. (2004). Accumulation of pathogenesis-related (pr) 10/bet v 1 protein homologues in mulberry (morus bombycis koidz.) tree during winter. Plant Cell and Environment 27 (9): 1112-1121.

Uota M. (1957). Preliminary study on storage of emperor grapes in controlled atmospheres with and without sulfur dioxide fumigation. Proceedings of American Society Horticulturae Science 69: 250–253.

Valero D., Valverde J.M., Martinez-Romero D., Guillen F., Castillo S., Serrano M. (2006). The combination of modified atmosphere packaging with eugenol or thymol to maintain quality, safety and functional properties of table grapes. Postharvest Biology and Technology 41: 317-327.

Vallejo L.F., Rinas U. (2004). Strategy for recovery of active protein through refolding of bacterial inclusion body proteins. Microbial Cell Factories 3: 2-12.

Valliyodan B., Nguyen H.T. (2006). Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Current Opinion in Plant Biology 9: 189-195.

Vamosvigyazo L. (1981). Polyphenol oxidase and peroxidase in fruits and vegetables. Crc Critical Reviews in Food Science and Nutrition 15: 49-127.

Van Buskirk H.A., Thomashow M.F. (2006). *Arabidopsis* transcription factors regulating cold acclimation. Physiologia Plantarum 126: 72–80.

Van de Rhee M.D., Lemmers R., Bol J.F. (1993). Analysis of regulatory elements involved in stress-induced and organ-specific expression of tobacco acidic and basic β-1,3-glucanse genes. Plant Molecular Biology 21: 451-461.

Van der Sluis A.A., Dekker M., De Jager A., et al. (2001). Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. Journal of Agricultural and Food Chemistry 49 (8): 3606-3613.

Van Loon L.C., Pierpont W.S., Boller T., Conjero V. (1994). Recommendations for naming plant pathogenesis-related proteins. Plant Molecular and Biology Report 12: 245–265.

Van Loon L.C., Rep M., Pieterse C.M.J. (2006). Significance of inducible defense-related proteins in infected plants. Annual Review of Phytopathology 44:1–28; 135-162.

Van Loon L.C., Van Kammen A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Smsun" and "Samsun NN" II. Changes in protein constitution after infection with tobacco mosaic virus. Virology 40: 199-206.

Van Loon, L.C. (1975). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun NN" IV. Similarity to qualitative changes of specific proteins after infection with different viruses and their relationship to acquire resistance. Virology 67: 566-572.

Varghese J.N., Garret T.P.J., Colman P.M., Chen L., Hoj P.B., Fincher G.B. (1994). Three-dimensional structures of two plant β-glucan endohydrolases with distinct substrate specificities. Proceedings of the National Academy of Science in USA 91: 2785-2789.

Vela G., Leon D.M., Garcia H.S., et al. (2003). Polyphenoloxidase activity during ripening and chilling stress in 'Manila' mangoes. Journal of Horticultural Science and Biotechnology 78 (1): 104-107.

Velazhahan R., Samiyappan R., Vidhyasekaran P. (2000). Purification of an elicitor-inducible antifungal chitinase from suspension-cultured rice cells. Phytoparasitica 28: 131-139.

Versari A., Parpinello G.P., Tornielli G.B., Ferrarini R., Giulivo C. (2001). Stilbene compounds and stilbene synthase expression during ripening, wilting, and UV treatment in grape cv. Corvina. Journal Agriclture and Food Chemistry 49: 5531-5536.

Vick B.A., Zimmerman D.C. (1984) Biosynthesis of jasmonic acid by several plant species. Plant Physiology 75: 458–461.

Vögeli-Lange R., Fründt C., Heart C.M., Nagy F., Meins F.Jr. (1994). Developmental, hormonal, and pathogenesis-related regulation of the tobacco class I beta-1,3-glucanase B promoter. Plant Molecular Biology 25: 299-311.

Vogelsang R., Barz W. (1993). Purification, characterization and differential hormonal-regulation of a beta-1,3-glucanase and 2 chitinases from chickpea (*Cicer arietinum L.*). Planta 189 (1): 60-69.

Waditee R., Bhuiyan M.N.H., Rai V., Aoki K., Tanaka Y., Hibino T., Suzuki S., Takano J., Jagendorf A.T., Takabe T et al. (2005). Genes for direct methylation of glycine provide high levels of glycinebetaine and abiotic-stree tolerance in *Synechococcus* and *Arabidopsis*. Proceedings of the National Academy of Science U.S.A 102: 1318-1323.

Walter W.M., Epley D.G., McFeeters R.F. (1990). Effect of water-stress on stored pickling cucumbers. Journal of Agricultural and Food Chemistry 38 (12): 2185-2191.

Walton D.C. (1980). Biochemistry and Physiology of Abcisic Acid. Annual Review of Plant Physiology 31: 453-489.

Wang C.Y. (1987). Changes of polyamines and ethylene in cucumber seedlings in response to chilling stress. Physiologia Plantarum 69: 253-257.

Wang C.Y. (1995). Effect of temperature preconditioning on catalase, peroxidase, and superoxide dismutase in chilling zucchini squash. Postharvest Biology and Technology 1: 33-45.

Wang C.Y., Steffens G.L. (1985). Effect of paclobutrazol on water stress-induced ethylene biosynthesis and polyamine accumulation in apple seedling leaves. Phytochemistry 24: 2185-2190.

Wang H., Cao G.H., Prior R.L. (1997). Oxygen radical absorbing capacity of anthocyanins. Journal of Agricultural and Food Chemistry 45 (2): 304-309.

Wang H.B., Race E.J., Shrikhande A.J. (2003). Characterization of anthocyanins in grape juices by ion trap liquid chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 51 (7): 1839-1844.

Wang J., Wu W.S., Zu K.J., Fei J., Sun X.F., Lin J., Li X. F., Tang K.X. (2004). Isolation and characterization of as erine/threonine protein kinase SOS2 gene from *Brassica napus*. Cellular and Molecular Biology Letters 9: 465-473.

Wang S.Y., Lewers K.S. (2007). Antioxidant capacity and flavonoid content in wild strawberries journal of the american society for horticultural science 132 (5): 629-637.

Wang S.Y., Lin H.S. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. Journal of Agricultural and Food Chemistry 48 (2): 140-146.

Wang S.Y., Wang C.Y., Weelburn A.R. (1990). Role of ethylene under stress conditions. In: Stress responses in plants: Adaptation and acclimation mechanisms. Wiley-Liss, Inc.pp. 147-173.

Wang Y., Chen J.Y., Jiang Y.M., Lu W.J. (2007). Cloning and expression analysis of phenylalanine ammonia-lyase in relation to chilling tolerance in harvested banana fruit. Postharvest Biology and Technology 44 (1): 34-41.

Ward E.R., Uknes S.J., Williams S.C., Dincher S.S., Wiederhold D.L., Alexander D.C., Ahl-Goy P., Metraux J.-P., Ryals J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3: 1085-1092.

Watanabe T., Kobori K., Miyashita K., Fujii T., Sakai H., Uchida M., Tanaka H. (1993). Identification of Glutamic acid 204 and Aspartic Acid 200 in chitinase A1 of *Bacillus circulans*. Journal of Biological Chemistry 268: 18567–18572.

Waterkeyn L. (1967). Sur l'existence d'un "stade callosique" présenté par la paroi cellulaire, au cours de la cytocinèse. C.R. Academic Science of Paris (D) 265:1792–1794.

Watkins C.B., Picton S., Grierson D. (1990). Stimulation and inhibition of expression of ripening-related mRNAs in tomatoes as influenced by chilling temperatures. Journal of Plant Physiology 136:318-323.

Wendehenne D., Pugin A., Klessig D. F., Durner, J. (2001) Nitric oxide: comparative synthesis and signaling in animals and plant cells. Trends in Plant Science 6: 177-183.

Wendehenne D., Pugin A., Klessig D.F., et al. (2001). Nitric oxide: comparative synthesis and signaling in animal and plant cells. trends in plant science 6 (4): 177-183.

Whetten R., Sederoff R. (1995). Lignin Biosynthesis. Plant Cell 7: 1001-1013.

White A., Dunn M.A., Brown K., Hughes M.A. (1994). Comparative analysis of genomic sequence and expression of a lipid transfer protein gene family in winter barley. Journal of Experimental Botany 45: 1885-1892.

Whitsett T.G., Lamortiniere C.A. (2006). Genistein and resveratrol: mammary cancer chemoprevention and mechanisms of action in the rat. Expert Review of Anticancer Therapy 6 (12): 1699-1706.

Wiese W., Vornam B., Krause E., Kindl H. (1994). Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine DNA fragment. Plant Molecular Biology 26: 667-677.

Winkel-Shirley B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology 126 (2): 485-493.

Winkler A., Cook J., Lider J.A., Kliewer W.M. (1974). Development and composition of grapes. In: General Viticulture. University of California Press, Berkeley, Los Angeles, London. pp. 151-157.

Wisniewski M., Webb R., Balsamo R., Close T.J., Yu X.M., Griffith M., (1999). Purification, immunolocalization, cryoprotective, and antifreeze activity of PCA60: A dehydrin from peach (*Prunus persica*). Physiologia Plantarum 105: 600-608.

Worrall D., Hird D.L., Hodge R., Paul W., Draper J., Scott R. (1992). Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco. Plant Cell 4: 759-771.

Wu J.R., Browse J. (1995). Elevated levels of high-melting-point phosphatidylglycerols do not induce chilling sensitivity in an *Arabidopsis* mutant. Plant Cell 7 (1): 17-27.

Xiao H., Nassuth A. (2006). Stress- and development-induced expression of spliced and unspliced transcripts from two highly similar dehydrin 1 genes in *V. riparia* and *V. vinifera.* Plant Cell Report 25: 968-977.

Xiao H., Siddiqua M., Braybrook S., et al. (2006). Three grape CBF/DREB1 genes respond to low temperature, drought and abscisic acid. Plant Cell and Environment 29 (7): 1410-1421.

Xu Y., Chang P.L., Liu D., Narasimhan M.L., Raghothma K.G., Hasegawa P.M., Versan R.A. (1994). Plant defense genes are synergistically induced by ethylene and methyljasmonate. Plant Cell 6: 1077-1085.

Yahia E.M., Nelson K.E., Kader A.A. (1983). Postharvest quality and storage life of grapes as influenced by adding carbon monoxide to air or controlled atmospheres. Journal of American Society and Horticultural Science 108: 1067-1071.

Yahia E.M., Vazquez-Moreno L. (1993). Responses of mango to insecticidal oxygen and carbon dioxide atmospheres. Lebensmittel-Wissenschaft und Technologie 26: 42-48.

Yaish M.W.F., Doxey A.C., McConkey B.J., Moffatt B.A., Griffith M. (2006). Cold-active winter rye glucanases with ice-binding capacity. Plant Physiology 141 (4): 1459-1472.

Yamagami T., Taria T., Axo Y., Ishiguro M. (1998). Isolation and characterisation of chitinase isoforms from the bulbs of four species of the genus *Tulipa*. Bioscience Biotechnology and Biochemistry 62: 584–587.

Yamaguchi T., Nakayama K., Hayashi T., Tanaka Y., Koike S. (2002). Molecular cloning and Characterization of a novel β-1,3-glucanase gene from rice. Bioscience Biotechnology and Biochemistry 66 (6): 1403-1406.

Yamauchi N., Minamide T., Otaga K. (1975). Physiological and chemical studies on ascorbic acid of fruits and vegetables. II. Changes of ascorbic acid content during development of chilling injury. Journal of the Japanese Society for Horticultural Science 44: 303-307.

Yang S. F., Hoffman N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. Annual Review of Plant Physiology 35: 155-189.

Yang S.F., Hoffman N.E. (1984). Ethylene biosynthesis and its regulation in higher-plants. Annual Review of Plant Physiology and Plant Molecular Biology 35: 155-189. Zhu B.L., Chen T.H.H., Li P.H. (1995). Expression of 3 osmotin-like protein genes in response to osmotic-stress and fungal infection in potato. Plant Molecular Biology 28 (1): 17-26.

Yeh S., Moffatt B.A., Griffith M., Xiong F., Yang D.S.C, Wiseman S.B., Sarhan F., Danyluk J., Xue Y.Q., Hew C.L., Doherty-Kirby A., Lajoie G. (2000). Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiology 124: 1251-1263.

Yoshikawa H., Honda C., Kondo S. (2007). Effect of low-temperature stress on abscisic acid, jasmonates, and polyamines in apples. Plant Growth Regulation 52 (3): 199-206.

Yu X., Griffith M. (1999). Antifreeze proteins in winter rye leaves form oligomeric complexes. Plant Physiology 119: 1361-1369.

Yu X., Griffith M., Wiseman S. (2001). Ethylene induces antifreeze activity in winter rye leaves. Plant Physiology 126:1232-1240.

Yun D., D'Urzo M.P., Abad L., Takeda S., Salzman R., Chen Z., Lee H., Hasegawa P.M., Bressan R.A. (1996). Novel osmotically induced antifungal chitinases and bacterial expression of an active recombinant isoform. Plant Physiology 111: 1219-1225.

Zacarías L., Lafuente M.T., Marcos J.F., Saladie M., Dupille E. (2003). Regulation of ethylene biosynthesis during cold storage of the chilling-sensitive Fortune mandarin fruit. In: Vendrell M, Klee HJ, Pech JC, Romojaro F, eds. Biology and biotechnology of the plant hormone ethylene III. Amsterdam: IOS Press, pp. 112–117.

Zhang J., Cui S., Li J., Wei J., Kirkham M.B. (1995). Protoplasmic factors, antioxidant responses, and chilling resistance in maize. Plant Physiology and Biochemistry 33:567-575.

Zhang J.H., Huang W.D., Pan Q.H., et al. (2005). Improvement of chilling tolerance and accumulation of heat shock proteins in grape berries (*Vitis vinifera* cv. Jingxiu) by heat pretreatment. Postharvest Biology and Technology 38 (1): 80-90.

Zhou H.W., Ben-Arie R., Lurie S. (2000). Pectin esterase, polygalacturonase and gel formation in peach pectin fractions. Phytochemistry, Oxford v. 55 (3), pp. 191-195.

Zoffoli J.P., Latorre B.A., Rodríguez E.J., Aldunce P. (1999). Modified atmosphere packaging using chlorine gas generators to prevent *Botrytis cinerea* on table grapes. Postharvest Biology and Technology 15: 135-142.